(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 402 116 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 90306154.7

(22) Date of filing: 06.06.90

(51) Int. Cl.5: C07K 13/00, A61K 39/12, G01N 33/569, C12N 15/40, C12N 15/86, C12Q 1/68, C12Q 1/70

The microorganism(s) has (have) been deposited with D.S.M., Braunschweig, W. Germany under number DSM 5734.

(30) Priority: 06.06.89 EP 89305708

(43) Date of publication of application:
12.12.90 Bulletin 90/50

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(71) Applicant: IMMUNO Aktiengesellschaft für chemisch-medizinische Produkte
Industriestrasse 72
A-1220 Wien(AT)

(72) Inventor: Heinz, Franz X.
Josefstadterstrasse 51/1/35,
A-1080 Wien(AT)
Inventor: Mandl, Christian W.
Liefelderg. 47/41
A-1160 Wien(AT)
Inventor: Kunz, Christian
Naaffg. 71
A-1180 Wien(AT)
Inventor: Dorner, Friedrich
Peterlinig. 17
A-1230 Wien(AT)
Inventor: Bodemer, Walter
Hesseg. 32/31
A-1228 Wien(AT)
Inventor: Antoine, Gerhard
Hollunderweg 11
A-2301 Gross Enzersdorf(AT)

(74) Representative: Sheard, Andrew Gregory et al
Kilburn & Strode 30, John Street
London WC1N 2DD(GB)

(54) Proteins, vaccines and nucleic acids.

(57) The invention discloses the nucleotide sequence and amino acid sequence of the NS-1 protein of western subtype tick-borne-encephalitis (TBE) virus. The sequence has been deduced from cDNA clones. The NS-1 protein can be used as a constituent of TBE vaccine, as a diagnostic reagent or for other purposes. Nucleic acid coding for the NS-1 protein can be used for the generation of expression systems or for the preparation of a live vaccine.

EP 0 402 116 A1

PROTEINS, VACCINES AND NUCLEIC ACIDS

The present invention relates to vaccines and diagnostic agents for western subtype TBE virus, and to nucleic acid and proteins useful for such vaccines and agents.

Western subtype tick-borne encephalitis (TBE) virus is a member of the family flaviviridae, which are spherical lipid enveloped RNA viruses (Westaway et al, Intervirology 24, 183-192, 1985). The prototype virus is yellow fever virus. By definition, all flaviviruses are serologically related as revealed by hemagglutination-inhibition assays. By cross-neutralisation, however, the family can be subgrouped into several serocomplexes (DeMadrid and Porterfield, J. Gen. Virol. 23,, 91-96, 1974) comprising more closely related flaviviruses as opposed to serologically more distantly related viruses of different serocomplexes or ungrouped flaviviruses. TBE virus is a member of the so-called tick-borne serocomplex, which in addition also contains viruses termed Louping ill, Langat, Omsk hemorrhagic fever, Kyasanur Forest disease, and Negishi.

TBE virus strains could be further assigned to a Western (European) subtype which is primarily transmitted by Ixodes ricinus and a Far Eastern subtype with Ixodes persulcatus as its main vector (Clarke, 1964; Bull. WHO 31, 45-56).

This subtype differentiation was confirmed by competitive RIA and peptide mapping using limited proteolysis of the corresponding structural glycoproteins (Heinz and Kunz, 1981; J. Gen. Virol. 57, 263-274) as well as antigenic analysis using monoclonal antibodies (Heinz et al, Virology 126, 525-537, 1983).

Mature virus particles contain only 3 structural proteins, termed E, C, and M with approximate molecular weights of 50 to 60,000, 15000, and 7000, respectively.

The genome of flaviviruses consists of single-stranded RNA of about 11000 bases with mRNA polarity having a molecular weight of 4 x 10^6 daltons. Together with the C-protein, this RNA forms a spherical nucleocapsid which is surrounded by a lipid envelope associated with both the proteins E and M. Experiments using purified preparations of the E protein obtained after solubilization of the virus by detergents have revealed that E represents the viral hemagglutinin, ie, it causes agglutination of certain erythrocytes under appropriate conditions, which upon immunisation, induces hemagglutination-inhibiting, neutralising and protective antibodies, as well as immunity against challenge with live virulent virus (Heinz et al, Infect. Immun. 33, 250-257, 1981).

In addition to these structural proteins, several non-structural virus-specified proteins have been found in cells infected with certain flaviviruses.

The genome organisation of flaviviruses has recently been determined by cDNA cloning and sequencing of yellow fever, West Nile and Murray Valley encephalitis virus (Rice et al, Science 229, 726-733, 1985; Dalgarno et al, J. Mol. Biol. 187, 309-323, 1986; Castle et al, Virology 147, 227-236, 1985; Castle et al, Virology 149, 10-26, 1986; Wengler et al, Virology 147, 264-274, 1985). According to these analyses, the genome RNA of flaviviruses contains a single long open reading frame of about 11,000 nucleotides which codes for all structural and non-structural proteins.

The gene order established for YF virus and confirmed for several other flaviviruses is 5;C-prM-(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 3.C, prM(M) and E represent structural proteins found in immature (C, prM, E) and mature (C, M, E) virus particles, whereas the rest of the genome codes for non-structural proteins. The structural proteins and the non-structural proteins have been positively identified by amino-terminal sequence analysis and have thus been correlated with the corresponding genome segments.

It is presumed that translation of viral proteins initiates at the first AUG at the 5'-end of the RNA and proceeds until a stop codon is encountered at the 3'-end of the RNA. The formation of individual proteins is thought to occur by a series of specific proteolytic cleavage events involving cellular as well as virus-specified proteases.

About 60 different flaviviruses have been recognised up to now and about two-thirds of them are transmitted by the bite of infected arthropods, thus representing arthropod-borne (ARBO) viruses. Several flaviviruses are well known human pathogens and include yellow fever virus, dengue virus, Japanese encephalitis virus, or tick-borne encephalitis virus (Shope, in: "The Togaviruses", pp. 47-82, Academic Press, New York, 1980).

TBE virus is endemic in many European countries, Russia and China. The disease is well documented in some Central European countries such as Austria, Czechoslovakia and Hungary, and several hundred hospitalised cases are recorded each year. This represents a significant public health problem.

The disease can be effectively prevented by vaccination with a highly purified formalin-inactivated whole virus vaccine (Kunz et al, J Med. Virol 6, 103-109, 1980) which induces an immune response against the structural proteins of the virus. The principal disadvantage of this vaccine is that large volumes of infectious and potentially haz-

ardous virus suspensions have to be handled in the course of the manufacturing process. Thus extensive and expensive safety precautions are required.

Means were therefore sought for preparing a vaccine which overcame the above discussed disadvantages. EP-A-0284791 addressed the problem by providing a DNA molecule that comprises Western subtype TBE virus derived DNA, coding for at least a part of at least one structural protein, ie selected from the group comprising the proteins C, prM, M or E, of Western subtype TBE virus.

Such a DNA molecule corresponds to the single stranded RNA of Western subtype TBE virus, and proved suitable for providing genetic information for expressing a polypeptide which may be the entire protein C, prM, M or E of Western subtype TBE virus, as described above, or part of one of the above-mentioned proteins. Such a polypeptide can be used diagnostically or therapeutically for example in the preparation of a vaccine.

The sequence analysis also confirmed the differentiation from the Far Eastern subtype by revealing an amino acid sequence diversity of up to 14 (depending on the protein) as compared to the Far Eastern subtype (Yamshchikov and Pletnov, Nucleic Acid Res. 16 7750 (1988)).

Although a vaccine based on one or more recombinant structural proteins as in EP-A-0284791 may well represent an improvement over an inactivated whole virus vaccine from the manufacturing point of view, it shares with the inactivated whole virus vaccine the disadvantage that it does not contain non-structural proteins, which may contribute to a protective immune response.

The non-structural proteins have important functions for the life cycle of flaviviruses being involved in virus maturation, proteolytic processing and RNA replication. Although one of the non-structural proteins (NS 1) has been shown for certain flaviviruses other than TBE to be capable of inducing a protective immune response (Schlessinger et al, J. Gen. Virol 68 853-857 (1987); Zhang et al, J. Virol. 62 3027-3031 (1988)) in experimental animals, there has been no such disclosure for non-structural proteins of TBE.

It is known that subneutralizing concentrations of neutralising antibodies or other antibodies to epitopes in the flavivirus E protein can mediate antibody-dependent enhancement of infectivity in Fc-receptor-bearing cells (Porterfield, Cardosa; "Concepts in Viral Pathogenesis I", Chapter 17 p.117) which has been implicated in the pathogenesis of dengue hemorrhagic fever and dengue shock syndrome (Halstead, Science 239 476-481 (1988)). In certain cases it may therefore be advantageous to avoid the use of flavivirus E protein in vaccines or to even to use NS 1 as the only vaccine component and not to incorporate viral structural proteins.

An NS-1 protein of the Western subtype of TBE virus has now been identified by its sequence. This protein is a useful vaccine component and may conveniently be produced by recombinant DNA technology.

According to a first aspect of the invention, there is provided a peptide or polypeptide comprising at least part of the amino acid sequence of the NS-1 protein of Western subtype TBE virus.

If the peptide or polypeptide contains only part of the amino acid sequence of the whole protein, it is preferred that it contain an immunogenic portion. Preferably, however, the peptide or polypeptide has the amino acid sequence shown in Figure 4.

The present invention includes explicitly all amino acid sequences which differ from the amino acid sequence of a natural NS-1 sequence whether by mutations and/or transpositions which are in the normal range of natural variation of Western subtype TBE virus. These sequences therefore still have the essential properties (particularly the essential antigenic properties) of the NS-1 non-structural protein of Western subtype TBE virus.

Although it is preferred to produce the peptides or polypeptides of this invention by expression of appropriate nucleotide sequences of this invention, it is also possible to isolate or, preferably, chemically to synthesise the peptides or polypeptides of the invention. The peptides or polypeptides may therefore be provided in substantially pure form and/or substantially free of other substances with which they are naturally associated.

Peptides or polypeptides according to first aspect of this invention may be useful on their own or in conjuction with one or more appropriate excipients as diagnostic agents and as ingredients in the manufacture of vaccines. Preferably the peptides or polypeptides are comprised in a composition which can be used in the medical field.

According to a second aspect of the present invention there is provided a vaccine composition comprising a peptide or polypeptide in accordance with the first aspect of the invention and one or more additional pharmaceutically acceptable components. Such components may include a pharmaceutically acceptable carrier or adjuvant for the peptide or polypeptide. Other suitable components may include a buffer.

The vaccine carrier may be any suitable carrier and may comprise one or more adjuvants if necessary. In accordance with usual practice all vaccine compositions in accordance with this invention may be sterile.

A further, preferred use of vaccines containing antigenic peptides or polypeptides is for preparing specific immunoglobulins, for example monoclonal or polyclonal antibodies, which may be prepared

by methods known to those skilled in the art. Antibodies against the peptides and polypeptides may also be regarded as forming part of the invention.

As mentioned above, a composition comprising one or more of the peptides or polypeptides according to this invention may also be used as a diagnostic reagent.

The invention also relates to nucleic acid coding for peptides or polypeptides in accordance with the first aspect.

According to a third aspect of the invention, there is provided nucleic acid coding for at least part of the NS-1 protein of Western subtype TBE virus. The nucleic acid, which may and often will be recombinant nucleic acid, particularly recombinant DNA, can code for the whole, or substantially the whole of the NS-1 protein or if appropriate for part of it.

Not only are these DNA and RNA molecules useful for providing the peptides or polypeptides in accordance with the first aspect, they may also be used in preparing a live vaccine. Preferably the DNA sequences are combined with the DNA of Vaccinia virus which is well established as a live vaccine.

Apart from their use as live vaccines, the DNA or RNA sequences according to this invention are additionally suitable as probes for screening purposes.

At present no antiviral agents are available for the specific treatment of TBE patients. There are, however, several potential possibilities to interfere specifically with the viral life cycle including processes that involve non-structural proteins such as RNA replication, posttranslational proteolytic processing and virus assembly. Nucleic acid coding for the protein NS-1 or part of it would also be useful for this purpose. In addition to interference with functional activities of viral proteins it is also feasible to block the viral replication process by the use of anti-sense RNA.

There are a number of ways for preparing DNA molecules within the scope of this invention. One possibility of obtaining the DNA molecule is first to extract the viral RNA from Western subtype TBE virus and purify the RNA molecule, followed by transcription of this RNA template into a DNA molecule using reverse transcriptase. A further possibility is chemically to synthesise the DNA molecules according this invention, once the DNA sequence has been investigated.

As a preferred embodiment, this invention includes DNA molecules which hybridise to DNA molecules according to the first aspect, and particularly to a DNA sequence as shown in Figure 3 and/or a DNA sequence coding for a protein sequence as shown in Figure 4, under stringent conditions, eg selecting for at least 90% nucleotide sequence homology. DNA molecules of this preferred kind may still code for peptides being able to cause antibody responses, and furthermore, those DNA molecules are suitable as DNA probes.

By one embodiment of the present invention nucleic acid including the complete sequence of wild-type Western subtype TBE virus genome coding for the non-structural protein NS-1 is provided as well as DNA molecules derived from the genomic RNA coding for at least a part of the protein. DNA molecules within the scope of the invention may correspond to or be complementary with the single stranded RNA of Western subtype TBE virus, and are suitable for providing genetic information for expressing the whole NS-1 protein or one or more parts thereof which usefully be used as a vaccine component or for diagnostic and therapeutic purposes.

The present invention includes explicitly all DNA sequences which differ from the DNA molecules corresponding or complementary to a natural NS-1 RNA sequence whether by degeneration of the genetic code and/or mutations and/or transpositions, which are in the normal range of natural variation of Western subtype TBE virus. These sequences therefore still code for proteins having the essential properties (particularly the essential antigenic properties) of the NS- 1 non-structural protein of Western subtype TBE virus.

In a preferred embodiment of this invention, the claimed DNA molecules can be combined with additional DNA sequences.

These additional sequences may allow replication and expression of the DNA molecule in a cell culture. The most important DNA sequences for this purpose are those which function as promoters, enhancers, polyadenylation signals, and splicing signals. These additional DNA sequences may be combined with the DNA molecules in accordance with the first aspect of the invention according to standard procedures known to those skilled in the art.

The advantages discussed above for DNA molecules within the scope of the invention are also true for RNA molecules in the same way. RNA molecules according to this invention may be obtained by isolation and purification of Western subtype TBE virus RNA, or by recombinant RNA/DNA techniques. Furthermore, not only are RNA molecules obtained by purification of the Western subtype TBE virus included in this invention, but also RNA molecules which have been obtained by transcribing isolated and purified virus RNA into DNA by reverse transcriptase and subsequent transcription of DNA thus obtained into RNA again, or by RNA-dependent RNA transcription.

In the present invention, not only are the DNA

and the RNA molecules as described above provided, but also vectors, comprising as an insert a DNA or RNA molecule within the scope of the invention. According to a fourth aspect of the invention, there is provided a vector, which may be a cloning vector or an expression vector, comprising a nucleic acid sequence in accordance with the third aspect. The vector may for example be a plasmid, virus (for example an animal (eg vaccinia) virus or a phage) or cosmid. As is known in the art, such vectors generally comprise sequences which control replication and expression of the inserted RNA or DNA sequences; such control sequences may comprise a promoter and possibly additionally an enhancer, among other sequences.

According to a fourth aspect of the invention, there is provided a host cell containing a vector as described above. Many such cells may be provided in a cell culture.

The vectors are preferably contained in cell cultures in which the expression of the polypeptides coded for by the RNA or DNA sequences is according to this invention, the cell culture being preferably a mammalian cell culture. By using a mammalian cell culture, the most preferred conditions are provided for expression of a polypeptide which is intended to be used as a vaccine for preventing mammals from Western subtype TBE virus infections.

For a better understanding of the present invention, and to show how it may be put into effect, preferred embodiments of the present invention will now be described with reference to the accompanying drawings, in which:

FIGURE 1 shows a photograph of agarose gel electrophoresis of the undigested plasmid 85 17-6 (lane b), which contains the entire coding sequence for the NS1 protein. Lane c shows a BamHI digest of the same plasmid and the two lanes a contain 0.6Kb, 2Kb and 10Kb size markers.

FIGURE 2 shows the complete nucleotide sequence of insert 17-6, contained in plasmid BS 17-6.

FIGURE 3 shows the RNA sequence and the encoded amino acid sequence of insert 17-6. The position numbers relate to the full length genome of TBE virus.

FIGURE 4 shows the amino acid sequence of protein NS1 as derived from the sequence information of clone 17-6. Position numbers are counted from the first amino acid of protein NS1.

FIGURE 5, referred to in Example 10 below, shows a strategy for cloning NS1 into the prokaryotic expression vector pUC19S.

FIGURES 6A and 6B, also referred to in Example 10, show nucleotide sequences of clone pNS1387 at the 5′ and3′ termini. An NS1 insert is in frame with the bacterial lacZ gene. Expression is under the control of the lacZ operator/promoter system. In FIGURE 6A the general organisation of the construct is shown. In FIGURE 6B the DNA and protein sequences are indicated. In this construct the NS1 has 40 additional nucleotides corresponding to 14 amino acids from the lacZ gene and the pUC19S polylinker at its 5′-end. There are an additional 27 non-NS1 nucleotides (corresponding to 9 amino acids) at the 3′-end. Nucleotide positions relative to the TBE genome, taken from FIGURE 3, are indicated by asterisks.

FIGURE 7A, referred to in Example 11, shows a strategy for cloning NS1 with its authentic 5′ signal sequence into the prokaryotic expression vector pUC19S.

FIGURE 7B, also referred to in Example 11, shows nucleotide sequences of clone ptSNS1791 at the 5′ and 3′ termini. The NS1 insert is in frame with the bacterial lacZ gene expression under the control of the lacZ promotor system. In part I the general organization of the construct is shown. In part II DNA and protein sequences are indicated. In this construct the NS1 has 39 additional nucleotides corresponding to 13 amino acids from the lacZ gene and the pUC19S polylinker at its 5′-end. There are additional 30 non-NS1 nucleotides corresponding to 10 amino acids at the 3′-end. Nucleotides positions relative to the TBE genome, taken from FIGURE 3, are indicated by asterisks.

FIGURE 8A, referred to in Example 12, shows the cloning of the NS1 coding region into the transfer vector pTKgptF1s for recombination with vaccinia virus.

FIGURE 8B, also referred to in Example 12, shows the nucleotide sequences of clone pAPNS1338 at the 5′ and 3′ termini. The NS1 has 19 additional nucleotides corresponding to 7 amino acids in its expected translation product from the polylinker region at its 5′-end additional 30 nucleotides, corresponding to 10 amino acids, are found at the 3′-end. Asterisks indicate the nucleotide positions in the TBE Virus genome taken from FIGURE 3.

FIGURES 9A and 9B, referred to in Example 13, show the cloning of NS1 and its putative signal sequence by the polymerase chain reaction (PCR). FIGURE 8A shows a generalised strategy for the PCR. FIGURE 8B shows the sequence of chemically synthesised oligonucleotides used as primers for the DNA amplification. The asterisks indicate the nucleotide positions in the viral genome according to FIGURE 3.

FIGURE 10, also referred to in Example 13, shows an agarose gel showing the NS1 fragment synthesised by PCR. The agarose gel is stained with ethidium bromide. DNA is detected under u/v light. The arrow indicates the fragment which is about 1130 bp long.

FIGURE 11, also referred to in Example 13, shows the cloning of the NS1 coding region with its putative signal sequence after synthesis by PCR into the transfer vector pSC11-OrthDELTA0 for recombination with vaccinia virus.

FIGURE 12, also referred to in Example 13, shows the sequences of clone pSCtSNS1444 at the 3′ and 5′ termini. The authentic NS1 coding region, including its signal sequence, acquires in its expected translation product 12 additional nucleotides, corresponding to four amino acids, from the polylinker region at its 5′-end. An additional 47 nucleotides (ie 16 amino acids) are found at the 3′-end. Asterisks indicate the nucleotide positions in the TBE virus genome, taken from FIGURE 3.

FIGURE 13, also referred to in Example 13, shows an analysis of the plasmid pSCtSNS1444 by digestion with appropriate restriction endonucleases. The DNA patterns indicate the correct orientation of the inserted NS1 sequence into the pSC11-Orth vector and confirms the size of the fragments as deduced from the nucleotide sequence of clone 17-6.

FIGURE 14, referred to in Example 14, shows the cloning of NS1 with its putative signal sequence into the transfer vector pTKgptF3s, for recombination with vaccina virus.

FIGURE 15, also referred to in Example 14, shows the nucleotide sequence of the plasmid pTKtSNS1556 at the 5′ and 3′ termini. In this construct the NS1 has 24 additional nucleotides corresponding to eight amino acids in its expected translation product from the polylinker region at its 5′-end. An additional 26 nucleotides (ie 9 amino acids) are found at the 3′-end. Asterisks indicate the nucleotide positions in the TBE virus genome, taken from FIGURE 3.

FIGURE 16, referred to in Example 15, shows a southern blot analysis of NS1 vaccinia recombinants varecNS1444 and varecNS1556. CV-1 cells infected with recombinant vaccinia viruses at a multiplicity of 5 pfu/cell. Two days after infection total cellular DNA was purified, digested with the restriction endonuclease HindIII and separated on 1% agarose gel. The DNA was transferred onto a nitrocellulose filter and hybridized with the radioactively labelled plasmid pSCtSNS1444 as a probe for the detection of the inserted NS1 fragment and adjacent viral tk sequences. A comparison of the recombinants varecNS1556-112, -124, -122 and varecNS1444-121 with wildtype Vaccinia DNA (slot 4) shows the expected upshift of the HindIII fragment towards a higher molecular weight. Only varecNS1556-222 shows a minor band corresponding to the w.t. fragment. This indicates that the plaque isloate is not homogeneous. 1556-122 etc. are different plaque isolates from the same recombination experiment after three plaque purfications. As controls and as size markers, different digests of the plasmid pSCtSNS1444 were used (slots 6 and 7).

FIGURE 17, referred to in Example 16, shows an autoradiograph of a denaturing SDS gel showing the recombinant viruses varecNS1444 and varecNS1556 expressing NS1 protein. A protein with an expected molecular weight of about 48kD can be detected which is not found in wild type or varec1342 (expressing a different protein) infected cells. The very strong protein band migrating above the 97kD marker band represents β-galactosidase (116kD) which is expressed under the control of the P11 promotor in the pSC11-Orth derived recombinants varecNS1444 and varec1342.

Nucleic acid in accordance with the present invention may be prepared and the sequence determined by the following general procedures:

First, viral RNA may be extracted from Western subtype TBE virus or Western subtype TBE virus infected cells. Using this RNA as a template, a double stranded cDNA may be synthesised, for example by reverse transcriptase. By the use of recombinant DNA techniques this cDNA may be inserted into a vector DNA such as Escherichia coli plasmid DNA to yield a recombinant plasmid. Recombinant plasmids may be used to transform appropriate host cells such as E. coli strain HB101 for amplification of the plasmids or the expression of the corresponding proteins. Individual colonies with insert-containing plasmids may be identified by the mini-plasmid preparation method according to Birnboim and Doly (Nucleic Acids Research, 7, 1195-1204, 1979). The base sequence of the Western subtype virus specific DNA present in the recombinant plasmid can be determined by rapid DNA sequencing methods such as the dideoxy chain termination method.

A more detailed description of one particular process of producing cDNA, recombinant plasmids, cells transformed with these plasmids and of determining the nucleotide sequence is as follows: First, Western subtype TBE viral RNA may be obtained from purified virus. For this purpose, Western subtype TBE virus can be grown in primary chick embryo cells, concentrated by ultracentrifugation, and purified by two cycles of sucrose density gradient centrifugation. After solubilising the proteins with SDS in the presence of proteinase K and RNAse inhibitor, eg by incubating for 1 hour at 37°C, the RNA is extracted with, for example, phenol, and precipitated with ethanol. Using this RNA as a template, a DNA complementary to the virus RNA is then synthesised in vitro by a reverse transcriptase, eg from avian myeloblastosis virus, for instance by the method of Gubler and Hofmann (Gene 25, 263-269, 1983). Using primers, such as

hexanucleotide primers obtained from calf thymus DNA, the viral RNA is incubated under appropriate conditions, eg as described in the manual "cDNA synthesis system" from Amersham, England, with a reverse transcriptase together with deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidin triphosphate (dCTP) as substrates. The thus obtained cDNA.RNA hybrid is treated with RNAse H under defined conditions which generates nicks in the RNA of the hybrid molecule. E. coli DNA polymerase I can then be used efficiently to replace the RNA strand utilising the nicked RNA as primers. The double-stranded RNA thus obtained is deproteinised by phenol-chloroform extraction, precipitated with ethanol and remaining RNA fragments are removed by treatment with RNAse (eg in TE buffer, 37° C, 30min).

Cloning of the dsDNA is performed, eg by the use of synthetic linkers. For this purpose, the dsDNA is treated with the Klenow fragment of E. coli DNA polymerase I under appropriate conditions (Maniatis et al, Molecular Cloning, CSH 1982) to ensure a maximum amount of clonable blunt ends, followed by phenol extraction for deproteinisation. dsDNA is incubated under appropriate conditions with BamHI linkers in the presence of DNA ligase. The reaction mixture is loaded and run on a 1% agarose gel and different size classes of cDNA are cut out of the gel and purified, eg by electroelution. On the other hand, a plasmid DNA to be used as vector DNA, eg E. coli plasmid BLUESCRIPT SK-, is cut with the restriction endonuclease BamHI and dephosphorylated by the use of bacterial alkaline phosphatase. After digestion of the cDNA with BamHI it is mixed with the BamHI -cut vector DNA and incubated at appropriate conditions to allow for hybridisation of the complementary overhanging sequences at the ends of both DNA molecules and ligated by the use of T4-DNA ligase. The recombinant DNA molecule is used to transform a competent E. coli strain (eg E. coli XL 1-blue). Recombinants containing virus specific inserts are identified by colour selection. Plasmids are isolated by the mini-plasmid preparation method (Birnboim and Doly, Nucleic Acids Research 7, 1195-1204, 1979) and insert sizes are analysed by restriction enzyme analyses using BamHI and separation of the fragments obtained on 1% agarose gels.

The base sequence of these inserts is determined by the dideoxy chain termination method according to Sanger et al (PNAS USA, 74, 5463-5467, 1977). The sequence obtained is analysed for homologies with already known sequences of other flaviviruses (Rice et al, Science 229, 726-733, 1985; Dalgarno et al, J. Mol. Biol, 187, 309-323, 1986; Castle et al, Virology 147 227-236, 1985; Castle et al, Virology 149, 10-26, 1986; Wengler et al, Virology 147, 264-274, 1985; Yamshchikov and Pletnev Nucleic Acid Res. 16 7750, 1988) by the aid of computer programs in order to determine the location of the sequence under investigation in the total sequence of the genome RNA. Figure 2 shows the complete nucleotide sequence and Figure 3 the corresponding amino acid sequence of the NS1 region of the western subtype TBE virus genome.

The present invention therefore for the first time provides the nucleotide sequence of the gene coding for the NS1 protein of the Western subtype TBE virus and, as a consequence, also provides the amino acid sequence of this protein.

The base sequence in Figure 2 is a preferred example for a DNA which codes for polypeptides having the characteristics of the Western subtype TBE virus NS1 protein. Due to the degeneration of the genetic code, the same amino acid sequence may also be coded for by base triplets other than those shown in the Figure.

Within the scope of protection of this invention there are also sequences included which are altered by mutation, transposition and degradation, which nevertheless code for an amino acid sequence still having the essential antigenic characteristics of the NS1 protein.

In addition, the invention does not only relate to exactly the same amino acid sequence as that shown in Figure 3, which represents only an exemplary sequence of the NS1 sequence of a natural Western subtype TBE virus isolate. It is a well-known fact that the genomes of RNA viruses are subject to higher mutation frequencies than those found with DNA viruses or cellular genes due to the high error rate of RNA polymerases and the lack of proof-reading mechanisms (Holland et al, Science 215, 1577-1585, 1982; Reanney, Ann. Rev. Microbiol. 36, 47-73, 1982). Depending on the characteristics of the virus, these mutations may give rise to the rapid development of new virus types due to antigenic drift, as is the case with influenza virus (Both et al in: "The Origin of Pandemic Influenza Viruses", W.G. Laver (ed.), Elsevier, 1983). On the other hand, RNA viruses can be antigenically more stable under natural ecological conditions, presumably due to functional constraints which do not allow for extensive structural changes in antigenically active proteins. Nevertheless, a certain degree of variation has to be taken into account and can be demonstrated by sequence comparison of different natural isolates.

This can be done for example by sequencing the RNA of different isolates using the dideoxy chain termination method with synthetic oligonucleotides as primers which have been prepared according to the sequence of the prototype

isolate shown in Figure 2.

All sequences which, compared to the prototype sequences, show slight variations such as those found in other natural isolates of Western subtype TBE virus are included in this invention. Such variations may also be obtained by in vitro modification of the nucleic acid. The invention therefore includes all sequences which hybridise under stringent conditions, eg selecting for at least 90% nucleotide sequence homology with the sequences or parts of the sequences disclosed and which preferably code for proteins or peptides having the essential antigenic determinant characteristics of the Western subtype TBE virus structural proteins.

Insertion of the nucleotide sequence coding for a protein such as NS-1 into appropriate vector(s) and host cells for expression at high level is a powerful technology for the study of the structure and biological function(s) of the protein. The expression system to be used is always dependent on certain molecular characteristics e.g. post-translational modification of the protein molecule of interest.

Expression can be performed in prokaryotic or eukaryotic cell systems. For glycoproteins such as NS-1 expression would better be performed in a eukaryotic host cell capable of proper glycosylation.

A preferred expression system consists of vaccinia virus and a primate cell growing continuously in culture. This ensures, or helps to ensure, that correct post translational modifications and high level synthesis can be achieved.

Furthermore there is ample evidence that gene regulatory sequences of vaccinia virus can direct the synthesis of almost any foreign protein either using a "late phase" promoter or using a "constitutive" promoter active during the early and late phase of viral replication (Moss and Flexner, Ann.Rev.Immunol. 4 305-324 (1987). However, it has been reported that the stability of foreign proteins expressed in vaccinia virus-infected cells is strongly influenced by the promoter regulating the expression of the gene of interest (Coupar et al., Eur.J.Immunol. 16 1479 (1986); Townsend et al., J.Exp-ivied. 168 1211-1224 (1988)).

The construction of appropriate expression plasmids containing NS-1 specific inserts requires extensive DNA manipulation. It has to be taken into account that NS-1 is part of a polyprotein which is post-translationally processed into individual proteins by cellular and viral protcases (Mandl et al., Virology 173 291-301 (1989)). In the course of a natural virus infection the synthesis of NS-1 involves an internal signal sequence at the NH_2-terminus. The signal directs the nascent polypeptide chan into the lumen of the endoplasmic reticulum, which is necessary for proper post-translational modifications such as glycosylation. In order to obtain a correctly processed expression product, this or a heterologous signal sequence has to be included in the recombinant molecule.

Furthermore, since NS-1 is part of a polyprotein it does not contain start and stop codons for translation. These have to be provided by specific recombinant DNA manipluations.

Hydrophobic (5′) signal and (3′) anchor sequences are included in the sequence of clone 17-6 (Figure 3). The lack of appropriate endonuclease restriction sites does not allow straightforward cloning of NS-1 with its "natural" signal sequence into insertion vectors. Therefore, a stretch of 1200 bp comprising the NS-1 signal sequence and the sequence of the mature NS-1 may be synthesised, for example, in a polymerase chain reaction (PCR). This technique also allows the introduction of optional restriction sites on both sides of the amplified DNA, as well as start and signal sequences.

A specifically designed primer-pair for the amplification results in an NS-1 sequence product flanked by recognition sites for the restriction endonuclease EcoRI. Using this methodology it is possible to exchange the authentic NS-1 signal sequence by any signal sequence naturally occuring or by a synthetic consensus signal sequence according to the proposals by v.Heinje (NAR 14 4683-4690 (1986)).

All plasmids, specifically developed for the expression of NS-1 molecules, can be transposed into a parental vaccinia virus (strain WR or attenuated derivates of the same) by homologous recombination in permissive host cell e.g. primate CV-1 cells. The experimental procedure to generate recombinant vaccinia viruses is common knowledge, is well known to those skilled in the art and does not need to be described (Mackett et al. JRL Press; "DNA cloning II" Ed. DM Glover 191-211, 1985).

The present invention also relates to the synthesis of NS-1 molecules in a bacterial host. Glycoproteins expressed in bacterial cells are usually not glycosylated in the same manner as in eukaryotic cells. Nevertheless synthesis of NS-1, even in a non-glycosylated form, may be useful for different purposes. As an example, derivatives of the envelope glycoprotein (gp) 160 of HIV-1 expressed in bacterial cells have significantly contributed to HIV-diagnosis and experimental vaccination.

Analogous to the construction of insertion plasmids for recombination with vaccinia virus an NS-1 sequence including its authentic signal sequence is synthesised with the PCR and cloned into, for example, pUC-19. A substitution of the TBE signal sequence by a prokaryotic signal sequence in or-

der to obtain efficient secretion of NS-1 molecules from the bacterial cells is possible. The latter may prevent the aggregation of NS-1 and formation of inclusion bodies that have to be dissolved with detergents or other solubilising agents.

Besides the synthesis of complete and mature NS-1 this experimental protocol can also be applied to truncated forms of the NS-1 molecule. Truncated molecules can be used to induce an immune response directed to specific sites of the protein. Such forms can also be valuable tools for performing "site directed serology". Immunologically relevant epitopes within the NS-1 may become incorporated into carrier molecules as adjuvants (e.g. HB-core particles) or into an immunstimulating complex (ISCOM) (Morein et al. Immunology Today 8(11) 333-338 (1987)).

Any of the NS-1 molecules synthesised in prokaryotic and eukaryotic expression systems described in this application represent molecules suitable as candidate NS-1 vaccine. either alone or perhaps by combining it with the recombinant glycoprotein-E of TBE-virus (EP-A-0284791) or the already available vaccine consisting of inactivated TBE-virus.

Further, the analysis of the immune response against this non-structural protein or parts thereof may be critical for understanding the pathogenicity of the TBE-virus and other members of the flavivirus family.

Foreign DNA or RNA sequences can also be engineered into the genomes of live viruses thus generating recombinant viruses which can be used as a live vaccine (for review, see Mackett and Smith, J. Gen. Virol. 67, 2067-2082, 1986).

Also combinations of different genes, eg derived from different viruses, can be simultaneously expressed by recombinant DNA technologies and used for vaccination (Perkus et al, Science, 229, 981-984, 1985). The invention therefore includes any combinations of sequences out of the sequences disclosed with other sequences, such as genes coding for other proteins or sequences contributing to the expression of the proteins, such as promoters, enhancers, polyadenylation or splicing signals.

It is well known to those skilled in the art that not necessarily the whole naturally occurring proteins have to be used for immunisation or diagnostic purposes (Lerner, Nature 299, 592-596, 1982; Arnon, TIBS 11, 521-524, 1986).

Proteins or parts of proteins to be used as vaccines or diagnostic reagents can not only be prepared by the recombinant DNA technologies described above, but the sequence information disclosed in the present invention can also be used for the chemical synthesis of oligopeptides. There is extensive literature available on this subject: peptides synthesised according to DNA sequences coding for many different proteins have been prepared and used for a variety of purposes such as molecular biological and immunological studies (Lerner et al, Cell, 23, 309-310, 1981; Lerner, Nature, 299, 592-596, 1982) or vaccination (Shinnick et al, Ann. Rev. Microbiol. 37, 425-446, 1983; DiMarchi et al, Science, 232, 639-641, 1986). The preparation and use of peptides or combinations of peptides corresponding to sequences disclosed in the present invention therefore represent the state of the art.

It is further known to those skilled in the art to use the nucleic acids and sequences provided by the present invention for the preparation of hybridisation probes eg for the use of determining virus RNA in ticks or blood-fluid (Meinkoth and Wahl, Anal. Biochem. 138, 267-284, 1984; Kulski and Norval, Arch. Virol. 83, 3-15, 1985). These can be prepared either by recombinant DNA technologies or by the chemical synthesis of oligonucleotides according to the sequence disclosed.

The invention will now be illustrated by the following examples. Where not specifically stated, procedures used follow those conventionally used in the art. Such procedures may be found in a number of standard works, including Sambrook, Fritsch and Maniatis "Molecular Cloning: A Laboratory Manual" (second edition), Cold Spring Harbor Laboratory Press, 1989.

EXAMPLES

Example 1 Propagation and Purification of TBE Virus

A 10% suspension of TBE virus-infected suckling mouse brain was used for the infection of primary chick embryo cell monolayers maintained in minimum essential medium (MEM) buffered with 15mM HEPES and 15mMEPPS at pH 7.6. After 40h incubation at 37°C the supernatant was clarified at 10000g for 30min at 4°C and the virus was pelleted by ultracentrifugation at 50000g for 3h at 4°C. The virus was then resuspended in an appropriate volume of TAN buffer (0.05M triethanolamine, 0.1M NaCl, pH 8.0) and subjected to rate-zonal centrifugation in a 5-20% (w/w) sucrose density gradient at 170000xg for 110min at 4°C. The virus peak was located by scanning the gradient at 254nm and subjected to equilibrium density gradient centrifugation in a 20 to 50% (w/w) sucrose gradient for 18 h/4°C at 150000xg. The virus peak was dialysed against TAN pH 8.0 to remove excess sucrose.

Example 2 Preparation of Viral RNA

100µg of purified virus were diluted into 400µl of proteinase K reaction buffer (10mM Tris pH 7.8, 5mM EDTA, 0.5% w/v SDS). Proteinase K was added to a final concentration of 200µg/ml and the mixture was incubated for 1hr at 37°C. Subsequently the solution was deproteinized by extracting it twice with equal volumes (400µl) of phenol and once with chloroform: isoamyl alcohol (24:1). then 26µl of a 3M Na-Acetate solution were added and the RNA was precipitated with 2.5 volumes of ethanol. Before further use, an aliquot of the RNA was denatured with glyoxal and run on a 1% agarose gel in order to check the yield and the quality of the preparation.

Example 3 Synthesis of Double Stranded cDNA

5µg of ethanol precipitated RNA were resuspended in 40µl of first strand synthesis buffer (Amersham, UK) that contained 5µg of random oligonucleotide primers, heated to 70°C for one minute and then allowed to cool slowly to room temperature. All four deoxynucleotide triphosphates were added to final concentrations of 1mM. Furthermore, 5 units of human placental ribonuclease inhibitor and 10µCi of α-^{32}P dCTP were added to the mixture. First strand synthesis was started by the addition of 100 units of reverse transcriptase (Amersham) and allowed to proceed for 2 hours at 42°C. Then the reaction mixture was placed on ice and the reagents for the second strand synthesis were added in this order: 93.5µl of second strand synthesis buffer (Amersham, UK), 4 units of ribonuclease H, 23 units of E. coli DNA polymerase and water to a final volume of 250µl. Second strand synthesis was carried out by subsequent incubations at 12°C and 22°C (2 hours each) and stopped by heating the solution for 20 minutes to 70°C. The double stranded cDNA was digested with 10 units of T4 DNA polymerase for 30 minutes at 37°C in order to create blunt ends. Finally the cDNA was purified by phenol and chloroform extractions and precipitated with 2 volumes of ethanol.

Example 4 Linker Ligation

Synthetic 5'-phosphorylated BamHI linkers (New England Biolabs) were added onto the cDNA in an overnight reaction at 12°C. The reaction mixture contained in a final volume of 20µl of ligation buffer (50mM Tris pH 7.5, 5mM $MgCl_2$, 5mM DTT, 1mM ATP) approximately 1µg of cDNA, 1µg of linker DNA and 1µl of T4 DNA ligase (New England Biolabs).

Example 5 Size Fractionation of cDNA

The cDNA was fractionated on a 1% agarose gel. Different size fractions were cut out of the gel and the DNA was extracted from the agarose in an analytical electroeluter (IBI) according to the manufacturer's directions. In spite of the small amount of DNA handled, which could hardly be detected by ethidium bromide staining, the extraction procedure could easily be monitored due to the radioactive label incorporated into the cDNA. The size fractionation step provided a means of selectively cloning large fragments of cDNA and also served to completely separate the cDNA from unligated linker molecules.

Example 6 Cloning into a Bacterial Phagemid

The cDNA was digested with 10U of BamHI restriction endonuclease for 1hr at 37°C. After phenol and chloroform extractions the DNA was precipitated with 2 volumes of ethanol and resuspended in a small volume of water. 20 - 30ng of the cDNA were mixed with 50ng of the phagemid BLUESCRIPT SK- (Stratagene) that had been linearized by BamHI digestion. (The word BLUESCRIPT is a trade mark.) These two components were litigated by T4 DNA ligase in 10µl of ligation buffer (see above) in an overnight reaction at 16°C. The next morning, the ligation mixture was heated to 65°C for 5min, diluted hundredfold with water and used to transform E. coli XL1 Blue cells. Competent bacteria were purchased from Stratagene and transformed with 3µl of the diluted ligation mixture exactly following the protocol provided by the manufacturer. Bacteria were plated onto LB agar plates containing ampicillin, tetracyclin, IPTG and X-Gal.

Example 7 Identification of Insert-Containing Phagemids

In the BLUESCRIPT vector system insert-containing phagemids yield white bacterial colonies, whereas self-ligated phagemid induces a blue colour reaction. Thus white colonies were picked and used to inoculate 2ml of LB-Medium containing ampicillin and grown overnight at 37°C in a shaker at 220 rotations per minute. The next day a quick plasmid preparation was performed by a standard boiling method (Birnboim and Doly, Nucleic Acids Research 7, 1195-1204, 1979). Plasmids were digested with the restriction enzyme BamHI and an-

alysed on 1% agarose gels. One clone identified in this manner was phagemid BS 17-6, that carries an approximately 2000 bp long virus specific insert. Figure 1 shows a 1% agarose gel of a quick plasmid preparation of BS 17-6 both in the supercoiled circular and the BamHI digested forms.

Example 8 Sequence Analysis of the Insert of Clone BS 17-6

Single stranded DNA of the clone BS 17-6 was prepared by addition of the VCS helper phage (Stratagene) at a M.O.I. of 20:1 to exponentially growing cultures of 85 17-6 containing E. coli XL1 Blue bacteria. After agitating vigorously the culture at 37°C for 16 hrs ssDNA was purified from the supernatant according to standard protocolls. This single-stranded DNA was sequenced in the whole region of the insert by the dideoxy method of Sanger et al (PNAS USA 74, 5463-5467; 1977) using the SEQUENASE enzyme (United States Biochemicals) and the reagents supplied by the enzyme-manufacturer. (The word SEQUENASE is a trade mark.) A vector specific and a set of virus-sequence specific oligonucleotides were used as primers for the sequencing reactions. The complete sequence of the insert 17-6 is shown in Figure 2.

Example 9 Derivation of the Amino Acid Sequence of Protein NS1

Translation of the nucleotide sequence of 17-6 in all possible reading frames revealed only one long open reading frame. The amino acid sequence derived in this frame was aligned to sequences of other flaviviruses (Rice et al, Science 229; 726-733 (1985); Coia et al, J. Gen. Virol. 69, 1-21 (1988); Chambers et al, Virology 169, 100-109 (1989)). For these analyses the Beckman MICROGENIE Software (Version 4.0) was employed on an IBM personal computer. The word MICROGENIE is a trade mark. The analyses indicated that clone 17-6 contained the entire coding sequence for protein NS1 and parts of the coding sequences of the proteins E and NS2A which flank the NS1 gene within the flaviviral genome.

Figure 3 depicts the RNA sequence and the encoded amino acid sequence derived from clone 17-6. The amino terminus of NS1 is supposed to be released by a cellular signalase, the carboxy-terminus by a signalase-like enzyme. A larger form of the NS1 protein that contains also the amino-terminal part of the protein NS2A was observed by Mason et al (Virology 158, 361-372 (1987)). The carboxy-terminus of this form of the NS1 protein may be liberated by a virus-specified protease or the cellular signalase (Rice et al, Science 229, 726-733 (1985)). The amino-terminus and three possible carboxy-termini are indicated in Figure 3. The carboxy-terminus liberated by a signalase-like enzyme (Chambers et al., Virology 169 100-109 (1988)) is depicted as "COOH-Terminus 1"; the carboxy-terminus possibly formed by a probably virus-specified protease is indicated as "COOH-Terminus 2"; and the potential carboxy terminus liberated by the cellular signalase is shown as "COOH-Terminus 3". The position numbers used in Figure 3 relate to the full length genome of TBE virus.

Figure 4 shows the amino acid sequence of protein NS1 as derived from the sequence information of clone 17-6. The possible COOH-Termini are indicated as in Figure 3. Position numbers are counted from the first amino acid of protein NS1. Figure 4 also depicts the locations of the three potential N-glycosylation sites present in the TBE virus protein NS1.

Example 10 Cloning of the NS1 coding region into a prokaryotic expression vector

The nucleotide sequence coding for NS1 is subcloned from clone 17-6 into pUC19. The vector pUC19S is a derivative of pUC19S containing a stop linker with a TAG stop codon in all three reading frames cloned into the BamHI site. The NS1 sequence containing a small part of NS2a at its 3'-end is excised with restriction endonuclease CfoI and blunt ends are generated with S1-Nuclease. The cloning vector pUC19S is linearized in its polylinker with SalI and termini are filled-in with T4 DNA Polymerase (Figure 5). Ligation of vector and insert regenerates the SalI sites and fuses NS1 in frame to the lacZ gene (Figures 6A and 6B). At the 3'terminus the NS1 nucleotide sequence is flanked by the prokaryotic transcriptional and translational stop signals contained in the vector. The plasmid is designated pNS1387.

Example 11 Cloning of the NS1 Coding Region including its 5' proximal hydrophobic signal sequence using the Polymerase Chain Rection

In order to construct a induceable prokaryotic expression plasmid containing the NS1 coding region including its authentic signal sequence the SalI fragment from pSCtSNS1444 (Example 13) is transferred to pUC19S resulting in clone ptSNS1682. To place the NS1 gene into the same reading frame as lacZ, clone ptSNS1682 is digested with HindIII, blunt ends are generated with

S1 Nuclease and religated thus generating clone ptSNS1791 with a deletion of 4 nucleotides (Figures 7a and 7b).

Example 12 Construction of a plasmid for recombination into Vaccinia Virus: the NS-1 protein under the control of the VV-p11 (L) promotor)

The NS-1 coding region (eg. in Example 10) is subcloned into the Sal I site of plasmid pTkgptF1s (Falkner, G.F. and B. Moss, (J. Virol 62,1849-1854 (1988)). The NS1 sequence is positioned in frame with the ATG translational start codon of the vector. Translational stop signals are also provided by the vector. The polylinker region of the vector adds an additional 7 aminoacids at the 5'end and 10 aminoacids at the 3'end. The cloning strategy and the flanking sequences of the protein are shown in Figure 8a and 8b. The plasmid is designated pAPNS1338.

Example 13 Construction of a plasmid for recombination into Vaccinia Virus the NS-1 protein with its natural signal sequence under the control of the VV-p7.5 (E/L) promotor

A nucleotide sequence comprising the NS1 coding region and its signal sequence is synthesized by the Polymerase Chain Reaction using clone 17-6 as a template. Two oligonucleotides with the restriction endonuclease cleavage sites EcoRI and SalI at the 5'end and SalI and HpaI at the 3'end are chemically synthesized and used as primers in PCR (Figs 9A and 9B). The amplified NS1 fragment is purified from an agarose gel (Fig. 10) cleaved with SalI and cloned into the SalI site of the vector pSC11-Orth after minor modifications. The plasmid pSC11-Orth has been deposited at the D.S.M., Braunschweig, West Germany, as DSM 5734 on 15th January 1990. The cloning strategy and sequences of the 5' and 3' termini of the new construct are shown in Figures 11 and 12. An agarose gel of a restriction digest of the clone confirming the correct orientation of the insert is shown in Figure 13. The plasmid is designated pSCtSNS1444.

Example 14 Construction of a plasmid for recombination into vaccinia virus: the NS-1 protein with its original signal sequence under the control of the VV-p11 (L) promotor

Plasmid pTKtSNS1556 results from subcloning of NS1 from clone pSCtSNS1444 into the vector pTKgptF3s (Falkner and Moss (J. Virol 62,1849-1854 (1988)). The NS1 sequence is exised with SalI and inserted into the SalI site of pTKgptF3s. The cloning strategy and nucleotide sequences of pTKtSNS1556 are shown in Figures 14 and 15.

All plasmids are grown under ampicillin selection of 100 µg/ml in E.coli hosts like DK1, BH101 or JM strains (JM105, JM83).

Example 15 Generation of Vaccinia Recombinants for Expression to NS1 protein

Recombinant viruses are generated according to standard procedures (Mackett, M., Smith, G. and Moss, B. In: DNA Cloning: A Practical Approach, pp 191-121. Edited by D.M. Glover; Oxford: IRL Press). The plasmid DNA of pSCtSNS14444 and of pTKtSNS1556 are transfected into CV-1 cells infectedwith vaccinia virus wild type. The pSC11-Orth-derived recombinant varecNs1444 is selected for with BuDR on 143tk-cells. Th pTKgptFs derived recombinant varecNS1556 is isolated by gpt selection on CV-1 cells. All recombinants were plaque purified three times. The correct organization of the recombinants is analysed by Southern blot analysis (Fig. 16).

Example 16 Expression of NS1 in CV-1 cells infected with Vaccinia virus recombinants

CV-1 cells are infected with the vaccinia virus recombinants varecNS1444 and varecNS1556. Two days after infection the cells are labelled with 35-S methionine for 4 hours. Proteins are separated on a 12% SDS denaturing polyacrylamide gel and identified by autoradiography. A protein band corresponding to an expected molecular weight of about 48 kD can be detected. It is missing in cells with wild type virus or non-NS1 recombinants like varec1342 (Fig. 17).

Claims

1. A peptide or polypeptide comprising at least part of the amino acid sequence of the NS-1 protein of Western subtype TBE virus.

2. A peptide or polypeptide as claimed in claim 1, which has the amino acid sequence shown in Figure 4.

3. NS-1 protein of Western subtype TBE virus.

4. A peptide or polypeptide comprising an amino acid sequence which differs from the amino acid sequence of a natural NS-1 sequence whether by mutations and/or transpositions which are in the normal range of natural variation of Western subtype TBE virus.

5. A peptide, polypeptide or protein as claimed in any one of claims 1 to 4 for use in medicine.

6. A vaccine composition comprising a peptide, polypeptide or protein as claimed in any one of claims 1 to 4 and one or more additional pharmaceutically acceptable components.

7. A diagnostic reagent comprising a peptide, polypeptide or protein as claimed in any one of claims 1 to 4.

8. Nucleic acid coding for a peptide, polypeptide or protein as claimed in any one of claims 1 to 4.

9. A live vaccine comprising nucleic acid as claimed in claim 8.

10. A probe comprising nucleic acid as claimed in claim 8.

11. Nucleic acid which hybridises to nucleic acid as claimed in claim 8.

12. Nucleic acid as claimed in claim 11 which hybridises to the DNA sequence shown in Figure 3.

13. Nucleic acid whose sequence differs from DNA molecules corresponding or complementary to a natural NS-1 RNA sequence whether by degeneracy of the genetic code and/or mutations and/or transpositions, which are in the normal range of natural variation of Western subtype TBE virus.

14. Nucleic acid as claimed in any one of claims 8 to 13 combined with additional nucleic acid sequences.

15. Nucleic acid as claimed in claim 14 which is DNA and wherein the additional sequences allow replication and expression of the DNA molecule in a cell culture and preferably comprise sequences which function as a promoter, an enhancer, a polyadenylation signal and/or a splicing signal.

16. A vector, such as a plasmid, virus or phage, including nucleic acid as claimed in any one of claims 8 to 15.

17. A vector as claimed in claim 16, which is derived from vaccinia virus.

18. A vector as claimed in claim 16, which is derived from a bacterial plasmid.

19. A host cell containing a vector as claimed in claim 16, 17 or 18.

20. A culture of host cells as claimed in claim 19.

21. A culture as claimed in claim 20 which is a mammalian cell culture.

Claims for the following Contracting State : ES

1. A process for preparing a peptide or polypeptide comprising at least part of the amino acid sequence of the NS-1 protein of Western subtype TBE virus, the process comprising coupling successive amino acids together.

2. A process as claimed in claim 1, wherein the peptide or polypeptide has the amino acid sequence shown in Figure 4.

3. A process for preparing NS-1 protein of Western subtype TBE virus, the process comprising coupling successive amino acids together.

4. A process for preparing a peptide or polypeptide comprising an amino acid sequence which differs from the amino acid sequence of a natural NS-1 sequence whether by mutations and/or transpositions which are in the normal range of natural variation of Western subtype TBE virus, the process comprising coupling successive amino acids together.

5. A peptide, polypeptide or protein, produceable by a process as claimed in any one of claims 1 to 4, for use in medicine.

6. A process for preparing a vaccine composition, the process comprising admixing a peptide, polypeptide or protein produceable by a process as claimed in any one of claims 1 to 4 and one or more additional pharmaceutically acceptable components.

7. A peptide, polypeptide or protein, produceable by a process as claimed in any one of claims 1 to 4, for use in diagnosis.

8. A process for preparing nucleic acid coding for a peptide, polypeptide or protein produceable by a process as claimed in any one of claims 1 to 4, the nucleic acid preparation process comprising coupling successive nucleotides together and/or linking oligo-and/or polynucleotides.

9. A process for preparing a live vaccine, the process comprising introducing nucleic acid produceable by a process as claimed in claim 8 into a suitable carrier.

10. A process as claimed in claim 8, which is a process for preparing a probe.

11. A process for preparing nucleic acid which hybridises to nucleic acid produceable by a process as claimed in claim 8, the process comprising coupling successive nucleotides together and/or linking oligo-and/or polynucleotides.

12. A process for preparing nucleic acid which hybridises to the DNA sequence shown in Figure 3, the process comprising coupling successive nucleotides together and/or linking oligo- and/or polynucleotides.

13. A process for preparing nucleic acid whose sequence differs from DNA molecules corresponding or complementary to a natural NS- 1 RNA sequence whether by degeneracy of the genetic code and/or mutations and/or transpositions, which are in the normal range of natural variation of Western subtype TBE virus, the process comprising coupling successive nucleotides together and/or linking oligo- and/or polynucleotides.

14. A process as claimed in any one of claims 8 to 13, wherein the nucleic acid produced com-

prises additional nucleic acid sequences.

15. A process as claimed in claim 14, wherein the nucleic acid is DNA and wherein the additional sequences allow replication and expression of the DNA molecule in a cell culture and preferably comprise sequences which function as a promoter, an enhancer, a polyadenylation signal and/or a splicing signal.

16. A process as claimed in any one of claims 8 to 15, wherein the nucleic acid is, or forms part of, a vector, such as a plasmid, virus or phage.

17. A process as claimed in claim 16, wherein the vector is derived from vaccinia virus.

18. A process as claimed in claim 16, wherein the vector is derived from a bacterial plasmid.

19. A host cell containing a vector produceable by a process as claimed in claim 16, 17 or 18.

20. A culture of host cells as claimed in claim 19.

21. A culture as claimed in claim 20 which is a mammalian cell culture.

Claims for the following Contracting State : GR

1. A process for preparing a peptide or polypeptide comprising at least part of the amino acid sequence of the NS-1 protein of Western subtype TBE virus, the process comprising coupling successive amino acids together.

2. A process as claimed in claim 1, wherein the peptide or polypeptide has the amino acid sequence shown in Figure 4.

3. A process for preparing NS-1 protein of Western subtype TBE virus, the process comprising coupling successive amino acids together.

4. A process for preparing a peptide or polypeptide comprising an amino acid sequence which differs from the amino acid sequence of a natural NS-1 sequence whether by mutations and/or transpositions which are in the normal range of natural variation of Western subtype TBE virus, the process comprising coupling successive amino acids together.

5. A peptide, polypeptide or protein, produceable by a process as claimed in any one of claims 1 to 4, for use in medicine.

6. A process for preparing a vaccine composition, the process comprising admixing a peptide, polypeptide or protein produceable by a process as claimed in any one of claims 1 to 4 and one or more additional pharmaceutically acceptable components.

7. A diagnostic reagent comprising a peptide, polypeptide or protein, produceable by a process as claimed in any one of claims 1 to 4.

8. Nucleic acid coding for a peptide, polypeptide or protein produceable by a process as claimed in any one of claims 1 to 4.

9. A process for producing a live vaccine, the process comprising introducing nucleic acid as claimed in claim 8 into a suitable carrier.

10. A probe comprising nucleic acid as claimed in claim 8.

11. Nucleic acid which hybridises to nucleic acid as claimed in claim 8.

12. Nucleic acid as claimed in claim 11 which hybridises to the DNA sequence shown in Figure 3.

13. Nucleic acid whose sequence differs from DNA molecules corresponding or complementary to a natural NS-1 RNA sequence whether by degeneracy of the genetic code and/or mutations and/or transpositions, which are in the normal range of natural variation of Western subtype TBE virus.

14. Nucleic acid as claimed in any one of claims 8 to 13 combined with additional nucleic acid sequences.

15. Nucleic acid as claimed in claim 14 which is DNA and wherein the additional sequences allow replication and expression of the DNA molecule in a cell culture and preferably comprise sequences which function as a promoter, an enhancer, a polyadenylation signal and/or a splicing signal.

16. A vector, such as a plasmid, virus or phage, including nucleic acid as claimed in any one of claims 8 to 15.

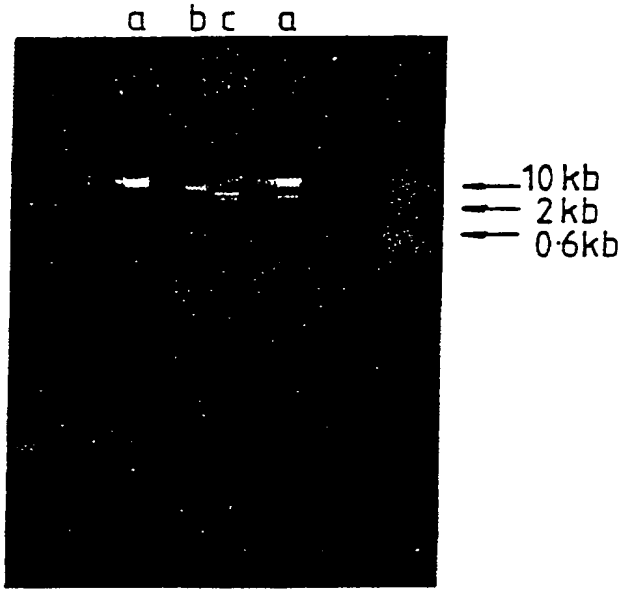
17. A vector as claimed in claim 16, which is derived from vaccinia virus.

18. A vector as claimed in claim 16, wherein the vector is derived from a bacterial plasmid.

19. A host cell containing a vector as claimed in claim 16, 17 or 18.

20. A culture of host cells as claimed in claim 19.

21. A culture as claimed in claim 20 which is a mammalian cell culture.



a = size marker (λ−DNA, HindIII digested)
b = BS 17−6 undigested
c = BS 17−6 BamHI digested:
upper band: BS SK−
lower band: 17−6 insert

Fig. 1.

```
        10          20          30          40          50          60
GGATCCCAGT CAGGGCAGTG GCACATGGAT CTCCAGATGT GAACGTGGCC ATGCTGATAA
CCTAGGGTCA GTCCCGTCAC CGTGTACCTA GAGGTCTACA CTTGCACCGG TACGACTATT

        70          80          90          100         110         120
CGCCAAACCC AACAATTGAA AACAATGGAG GTGGCTTCAT AGAGATGCAG CTGCCCCCAG
GCGGTTTGGG TTGTTAACTT TTGTTACCTC CACCGAAGTA TCTCTACGTC GACGGGGGTC

        130         140         150         160         170         180
GGGATAACAT CATCTATGTT GGGGAACTGA GTCATCAATG GTTCCAAAAA GGGAGCAGCA
CCCTATTGTA GTAGATACAA CCCCTTGACT CAGTAGTTAC CAAGGTTTTT CCCTCGTCGT

        190         200         210         220         230         240
TCGGAAGGGT TTTCCAAAAG ACCAAGAAAG GCATAGAAAG ACTGACAGTG ATAGGAGAGC
AGCCTTCCCA AAAGGTTTTC TGGTTCTTTC CGTATCTTTC TGACTGTCAC TATCCTCTCG

        250         260         270         280         290         300
ACGCCTGGGA CTTCGGTTCT GCTGGAGGCT TTCTGAGTTC AATTGGGAAG GCGGTACATA
TGCGGACCCT GAAGCCAAGA CGACCTCCGA AAGACTCAAG TTAACCCTTC CGCCATGTAT

        310         320         330         340         350         360
CGGTCCTTGG TGGCGCTTTC AACAGCATCT TCGGGGGAGT GGGGTTTCTA CCAAAACTTT
GCCAGGAACC ACCGCGAAAG TTGTCGTAGA AGCCCCCTCA CCCCAAAGAT GGTTTTGAAA

        370         380         390         400         410         420
TATTAGGAGT GGCATTGGCT TGGTTGGGCC TGAACATGAG AAACCCTACA ATGTCCATGA
ATAATCCTCA CCGTAACCGA ACCAACCCGG ACTTGTACTC TTTGGGATGT TACAGGTACT

        430         440         450         460         470         480
GCTTTCTCTT GGCTGGAGGT CTGGTCTTGG CCATGACCCT TGGAGTGGGG GCGGATGTTG
CGAAAGAGAA CCGACCTCCA GACCAGAACC GGTACTGGGA ACCTCACCCC CGCCTACAAC
```

Fig.2(i).

```
      490         500         510         520         530         540
GTTGCGCTGT GGACACGGAA CGAATGGAGC TCCGCTGTGG CGAGGGCCTG GTCGTGTGGA
CAACGCGACA CCTGTGCCTT GCTTACCTCG AGGCGACACC GCTCCCGGAC CAGCACACCT

      550         560         570         580         590         600
GAGAGGTCTC AGAATGGTAT GACAATTATG CCTACTACCC GGAGACACCG GGGGCCCTTG
CTCTCCAGAG TCTTACCATA CTGTTAATAC GGATGATGGG CCTCTGTGGC CCCCGGGAAC

      610         620         630         640         650         660
CATCAGCCAT AAAGGAGACA TTTGAGGAGG GAAGCTGTGG TGTAGTCCCC CAGAACAGGC
GTAGTCGGTA TTTCCTCTGT AAACTCCTCC CTTCGACACC ACATCAGGGG GTCTTGTCCG

      670         680         690         700         710         720
TCGAGATGGC CATGTGGAGA AGCTCGGTCA CAGAGCTGAA TTTGGCTCTG GCGGAAGGGG
AGCTCTACCG GTACACCTCT TCGAGCCAGT GTCTCGACTT AAACCGAGAC CGCCTTCCCC

      730         740         750         760         770         780
AGGCAAATCT CACAGTGGTG GTGGACAAGT TTGACCCCAC TGACTACCGA GGTGGTGTCC
TCCGTTTAGA GTGTCACCAC CACCTGTTCA AACTGGGGTG ACTGATGGCT CCACCACAGG

      790         800         810         820         830         840
CTGGTTTACT GAAAAAAGGA AAGGACATAA AAGTCTCCTG GAAAAGCTGG GGCCATTCAA
GACCAAATGA CTTTTTTCCT TTCCTGTATT TTCAGAGGAC CTTTTCGACC CCGGTAAGTT

      850         860         870         880         890         900
TGATCTGGAG CATTCCTGAG GCCCCCCGTC GCTTCATGGT GGGCACGGAA GGACAAAGTG
ACTAGACCTC GTAAGGACTC CGGGGGGCAG CGAAGTACCA CCCGTGCCTT CCTGTTTCAC

      910         920         930         940         950         960
AGTGTCCCCT AGAGAGACGG AAGACAGGTG TTTTCACGGT GGCAGAATTC GGGGTTGGCC
TCACAGGGGA TCTCTCTGCC TTCTGTCCAC AAAAGTGCCA CCGTCTTAAG CCCCAACCGG

      970         980         990        1000        1010        1020
TGAGAACAAA GGTCTTCTTG GATTTCAGAC AGGAACCAAC ACATGAGTGT GACACAGGAG
ACTCTTGTTT CCAGAAGAAC CTAAAGTCTG TCCTTGGTTG TGTACTCACA CTGTGTCCTC
```

Fig.2(ii).

```
      1030        1040        1050        1060        1070        1080
TGATGGGAGC TGCAGTCAAG AACGGCATGG CAATCCACAC AGATCAAAGT CTCTGGATGA
ACTACCCTCG ACGTCAGTTC TTGCCGTACC GTTAGGTGTG TCTAGTTTCA GAGACCTACT

      1090        1100        1110        1120        1130        1140
GATCAATGAA AAATGACACA GGCACTTACA TAGTTGAACT TTTGGTCACT GACCTGAGGA
CTAGTTACTT TTTACTGTGT CCGTGAATGT ATCAACTTGA AAACCAGTGA CTGGACTCCT

      1150        1160        1170        1180        1190        1200
ACTGCTCATG GCCTGCTAGC CACACTATCG ATAATGCTGA CGTGGTGGAC TCAGAGTTAT
TGACGAGTAC CGGACGATCG GTGTGATAGC TATTACGACT GCACCACCTG AGTCTCAATA

      1210        1220        1230        1240        1250        1260
TCCTTCCGGC GAGCCTGGCA GGACCCAGAT CCTGGTACAA CAGGATACCT GGCTATTCAG
AGGAAGGCCG CTCGGACCGT CCTGGGTCTA GGACCATGTT GTCCTATGGA CCGATAAGTC

      1270        1280        1290        1300        1310        1320
AACAGGTGAA AGGGCCATGG AAGTACACGC CTATCCGTGT TATCAGAGAG GAGTGTCCCG
TTGTCCACTT TCCCGGTACC TTCATGTGCG GATAGGCACA ATAGTCTCTC CTCACAGGGC

      1330        1340        1350        1360        1370        1380
GCACGACCGT TACCATCAAC GCCAAGTGTG ACAAAAGAGG AGCATCTGTG AGGAGTACCA
CGTGCTGGCA ATGGTAGTTG CGGTTCACAC TGTTTTCTCC TCGTAGACAC TCCTCATGGT

      1390        1400        1410        1420        1430        1440
CAGAGAGTGG CAAGGTTATC CCAGAATGGT GCTGCCGAGC GTGCACAATG CCACCAGTGA
GTCTCTCACC GTTCCAATAG GGTCTTACCA CGACGGCTCG CACGTGTTAC GGTGGTCACT

      1450        1460        1470        1480        1490        1500
CGTTCCGGAC TGGAACTGAT TGCTGGTATG CCATGGAAAT ACGGCCAGTC CATGACCAGG
GCAAGGCCTG ACCTTGACTA ACGACCATAC GGTACCTTTA TGCCGGTCAG GTACTGGTCC

      1510        1520        1530        1540        1550        1560
GGGGGCTTGT TCGCTCAATG GTGGTTGCGG ACAACGGTGA ATTACTTAGT GAGGGAGGAG
CCCCCGAACA AGCGAGTTAC CACCAACGCC TGTTGCCACT TAATGAATCA CTCCCTCCTC
```

Fig.2(iii).

```
      1570        1580        1590        1600        1610        1620
TCCCCGGAAT AGTGGCATTG TTTGTGGTCC TTGAATACAT CATCCGTAGG AGGCCCTCCA
AGGGGCCTTA TCACCGTAAC AAACACCAGG AACTTATGTA GTAGGCATCC TCCGGGAGGT

      1630        1640        1650        1660        1670        1680
CGGGAACGAC GGTTGTGTGG GGGGGTATCG TCGTTCTCGC TCTGCTTGTC ACCGGGATGG
GCCCTTGCTG CCAACACACC CCCCCATAGC AGCAAGAGCG AGACGAACAG TGGCCCTACC

      1690        1700        1710        1720        1730        1740
TCAGGATAGA GAGCCTGGTG CGCTATGTGG TGGCAGTGGG GATCACATTC CACCTTGAGC
AGTCCTATCT CTCGGACCAC GCGATACACC ACCGTCACCC CTAGTGTAAG GTGGAACTCG

      1750        1760        1770        1780        1790        1800
TGGGGCCAGA GATCGTGGCC TTGATGCTAC TCCAGGCTGT GTTTGAGCTG AGGGTGGGTT
ACCCCGGTCT CTAGCACCGG AACTACGATG AGGTCCGACA CAAACTCGAC TCCCACCCAA

      1810        1820        1830        1840        1850        1860
TGCTCAGCGC ATTTGCATTG CGCAGAAGCC TCACCGTCCG AGAGATGGTG ACCACCTACT
ACGAGTCGCG TAAACGTAAC GCGTCTTCGG AGTGGCAGGC TCTCTACCAC TGGTGGATGA

      1870        1880        1890        1900        1910        1920
TTCTTTTGCT GGTCCTGGAA TTGGGGCTGC CGGGTGCGAG CCTTGAGGAG TTCTGGAAAT
AAGAAAACGA CCAGGACCTT AACCCCGACG GCCCACGCTC GGAACTCCTC AAGACCTTTA

      1930        1940        1950
GGGGTGATGC ACTGGCCATG GGGGCGCCGC GGATCC
CCCCACTACG TGACCGGTAC CCCCGCGGCG CCTAGG
```

Fig.2(iv).

TBE(RNA) LIMITS: 1973 3916

2002 2032
AUC CCA GUC AGG GCA GUG GCA CAU GGA UCU CCA GAU GUG AAC GUG GCC AUG CUG AUA ACG
Ile Pro Val Arg Ala Val Ala His Gly Ser Pro Asp Val Asn Val Ala Met Leu Ile Thr

2062 2092
CCA AAC CCA ACA AUU GAA AAC AAU GGA GGU GGC UUC AUA GAG AUG CAG CUG CCC CCA GGG
Pro Asn Pro Thr Ile Glu Asn Asn Gly Gly Gly Phe Ile Glu Met Gln Leu Pro Pro Gly

2122 2152
GAU AAC AUC AUC UAU GUU GGG GAA CUG AGU CAU CAA UGG UUC CAA AAA GGG AGC AGC AUC
Asp Asn Ile Ile Tyr Val Gly Glu Leu Ser His Gln Trp Phe Gln Lys Gly Ser Ser Ile

2182 2212
GGA AGG GUU UUC CAA AAG ACC AAG AAA GGC AUA GAA AGA CUG ACA GUG AUA GGA GAG CAC
Gly Arg Val Phe Gln Lys Thr Lys Lys Gly Ile Glu Arg Leu Thr Val Ile Gly Glu His

2242 2272
GCC UGG GAC UUC GGU UCU GCU GGA GGC UUU CUG AGU UCA AUU GGG AAG GCG GUA CAU ACG
Ala Trp Asp Phe Gly Ser Ala Gly Gly Phe Leu Ser Ser Ile Gly Lys Ala Val His Thr

2302 2332
GUC CUU GGU GGC GCU UUC AAC AGC AUC UUC GGG GGA GUG GGG UUU CUA CCA AAA CUU UUA
Val Leu Gly Gly Ala Phe Asn Ser Ile Phe Gly Gly Val Gly Phe Leu Pro Lys Leu Leu

2362 2392
UUA GGA GUG GCA UUG GCU UGG UUG GGC CUG AAC AUG AGA AAC CCU ACA AUG UCC AUG AGC
Leu Gly Val Ala Leu Ala Trp Leu Gly Leu Asn Met Arg Asn Pro Thr Met Ser Met Ser

NH2-Terminus

2422 2452
UUU CUC UUG GCU GGA GGU CUG GUC UUG GCC AUG ACC CUU GGA GUG GGG GCG GAU GUU GGU
Phe Leu Leu Ala Gly Gly Leu Val Leu Ala Met Thr Leu Gly Val Gly Ala Asp Val Gly

2482 2512
UGC GCU GUG GAC ACG GAA CGA AUG GAG CUC CGC UGU GGC GAG GGC CUG GUC GUG UGG AGA
Cys Ala Val Asp Thr Glu Arg Met Glu Leu Arg Cys Gly Glu Gly Leu Val Val Trp Arg

2542 2572
GAG GUC UCA GAA UGG UAU GAC AAU UAU GCC UAC UAC CCG GAG ACA CCG GGG GCC CUU GCA
Glu Val Ser Glu Trp Tyr Asp Asn Tyr Ala Tyr Tyr Pro Glu Thr Pro Gly Ala Leu Ala

2602 2632
UCA GCC AUA AAG GAG ACA UUU GAG GAG GGA AGC UGU GGU GUA GUC CCC CAG AAC AGG CUC
Ser Ala Ile Lys Glu Thr Phe Glu Glu Gly Ser Cys Gly Val Val Pro Gln Asn Arg Leu

Fig.3(i).

2662 2692
GAG AUG GCC AUG UGG AGA AGC UCG GUC ACA GAG CUG AAU UUG GCU CUG GCG GAA GGG GAG
Glu Met Ala Met Trp Arg Ser Ser Val Thr Glu Leu Asn Leu Ala Leu Ala Glu Gly Glu

2722 2752
GCA AAU CUC ACA GUG GUG GUG GAC AAG UUU GAC CCC ACU GAC UAC CGA GGU GGU GUC CCU
Ala Asn Leu Thr Val Val Val Asp Lys Phe Asp Pro Thr Asp Tyr Arg Gly Gly Val Pro

2782 2812
GGU UUA CUG AAA AAA GGA AAG GAC AUA AAA GUC UCC UGG AAA AGC UGG GGC CAU UCA AUG
Gly Leu Leu Lys Lys Gly Lys Asp Ile Lys Val Ser Trp Lys Ser Trp Gly His Ser Met

2842 2872
AUC UGG AGC AUU CCU GAG GCC CCC CGU CGC UUC AUG GUG GGC ACG GAA GGA CAA AGU GAG
Ile Trp Ser Ile Pro Glu Ala Pro Arg Arg Phe Met Val Gly Thr Glu Gly Gln Ser Glu

2902 2932
UGU CCC CUA GAG AGA CGG AAG ACA GGU GUU UUC ACG GUG GCA GAA UUC GGG GUU GGC CUG
Cys Pro Leu Glu Arg Arg Lys Thr Gly Val Phe Thr Val Ala Glu Phe Gly Val Gly Leu

2962 2992
AGA ACA AAG GUC UUC UUG GAU UUC AGA CAG GAA CCA ACA CAU GAG UGU GAC ACA GGA GUG
Arg Thr Lys Val Phe Leu Asp Phe Arg Gln Glu Pro Thr His Glu Cys Asp Thr Gly Val

3022 3052
AUG GGA GCU GCA GUC AAG AAC GGC AUG GCA AUC CAC ACA GAU CAA AGU CUC UGG AUG AGA
Met Gly Ala Ala Val Lys Asn Gly Met Ala Ile His Thr Asp Gln Ser Leu Trp Met Arg

3082 3112
UCA AUG AAA AAU GAC ACA GGC ACU UAC AUA GUU GAA CUU UUG GUC ACU GAC CUG AGG AAC
Ser Met Lys Asn Asp Thr Gly Thr Tyr Ile Val Glu Leu Leu Val Thr Asp Leu Arg Asn

3142 3172
UGC UCA UGG CCU GCU AGC CAC ACU AUC GAU AAU GCU GAC GUG GUG GAC UCA GAG UUA UUC
Cys Ser Trp Pro Ala Ser His Thr Ile Asp Asn Ala Asp Val Val Asp Ser Glu Leu Phe

3202 3232
CUU CCG GCG AGC CUG GCA GGA CCC AGA UCC UGG UAC AAC AGG AUA CCU GGC UAU UCA GAA
Leu Pro Ala Ser Leu Ala Gly Pro Arg Ser Trp Tyr Asn Arg Ile Pro Gly Tyr Ser Glu

3262 3292
CAG GUG AAA GGG CCA UGG AAG UAC ACG CCU AUC CGU GUU AUC AGA GAG GAG UGU CCC GGC
Gln Val Lys Gly Pro Trp Lys Tyr Thr Pro Ile Arg Val Ile Arg Glu Glu Cys Pro Gly

Fig.3(ii).

```
                                    3322                                3352
ACG ACC GUU ACC AUC AAC GCC AAG UGU GAC AAA AGA GGA GCA UCU GUG AGG AGU ACC ACA
Thr Thr Val Thr Ile Asn Ala Lys Cys Asp Lys Arg Gly Ala Ser Val Arg Ser Thr Thr

                                    3382                                3412
GAG AGU GGC AAG GUU AUC CCA GAA UGG UGC UGC CGA GCG UGC ACA AUG CCA CCA GUG ACG
Glu Ser Gly Lys Val Ile Pro Glu Trp Cys Cys Arg Ala Cys Thr Met Pro Pro Val Thr

                                    3442                                3472
UUC CGG ACU GGA ACU GAU UGC UGG UAU GCC AUG GAA AUA CGG CCA GUC CAU GAC CAG GGG
Phe Arg Thr Gly Thr Asp Cys Trp Tyr Ala Met Glu Ile Arg Pro Val His Asp Gln Gly
                    COOH-Terminus 1
                                    3502                                3532
GGG CUU GUU CGC UCA AUG GUG GUU GCG GAC AAC GGU GAA UUA CUU AGU GAG GGA GGA GUC
Gly Leu Val Arg Ser Met Val Val Ala Asp Asn Gly Glu Leu Leu Ser Glu Gly Gly Val
                                                COOH-Terminus 2
                                    3562                                3592
CCC GGA AUA GUG GCA UUG UUU GUG GUC CUU GAA UAC AUC AUC CGU AGG AGG CCC UCC ACG
Pro Gly Ile Val Ala Leu Phe Val Val Leu Glu Tyr Ile Ile Arg Arg Arg Pro Ser Thr

                                    3622                                3652
GGA ACG ACG GUU GUG UGG GGG GGU AUC GUC GUU CUC GCU CUG CUU GUC ACC GGG AUG GUC
Gly Thr Thr Val Val Trp Gly Gly Ile Val Val Leu Ala Leu Leu Val Thr Gly Met Val
                            COOH-Terminus 3
                                    3682                                3712
AGG AUA GAG AGC CUG GUG CGC UAU GUG GUG GCA GUG GGG AUC ACA UUC CAC CUU GAG CUG
Arg Ile Glu Ser Leu Val Arg Tyr Val Val Ala Val Gly Ile Thr Phe His Leu Glu Leu

                                    3742                                3772
GGG CCA GAG AUC GUG GCC UUG AUG CUA CUC CAG GCU GUG UUU GAG CUG AGG GUG GGU UUG
Gly Pro Glu Ile Val Ala Leu Met Leu Leu Gln Ala Val Phe Glu Leu Arg Val Gly Leu

                                    3802                                3832
CUC AGC GCA UUU GCA UUG CGC AGA AGC CUC ACC GUC CGA GAG AUG GUG ACC ACC UAC UUU
Leu Ser Ala Phe Ala Leu Arg Arg Ser Leu Thr Val Arg Glu Met Val Thr Thr Tyr Phe

                                    3862                                3892
CUU UUG CUG GUC CUG GAA UUG GGG CUG CCG GGU GCG AGC CUU GAG GAG UUC UGG AAA UGG
Leu Leu Leu Val Leu Glu Leu Gly Leu Pro Gly Ala Ser Leu Glu Glu Phe Trp Lys Trp


GGU GAU GCA CUG GCC AUG GGG GCG
Gly Asp Ala Leu Ala Met Gly Ala
```

Fig.3(iii).

TBE-NS1

10 20
Asp Val Gly Cys Ala Val Asp Thr Glu Arg Met Glu Leu Arg Cys Gly Glu Gly Leu Val

30 40
Val Trp Arg Glu Val Ser Glu Trp Tyr Asp Asn Tyr Ala Tyr Tyr Pro Glu Thr Pro Gly

50 60
Ala Leu Ala Ser Ala Ile Lys Glu Thr Phe Glu Glu Gly Ser Cys Gly Val Val Pro Gln

70 80
Asn Arg Leu Glu Met Ala Met Trp Arg Ser Ser Val Thr Glu Leu Asn Leu Ala Leu Ala

90 100
Glu Gly Glu Ala Asn Leu Thr Val Val Val Asp Lys Phe Asp Pro Thr Asp Tyr Arg Gly

110 120
Gly Val Pro Gly Leu Leu Lys Lys Gly Lys Asp Ile Lys Val Ser Trp Lys Ser Trp Gly

130 140
His Ser Met Ile Trp Ser Ile Pro Glu Ala Pro Arg Arg Phe Met Val Gly Thr Glu Gly

150 160
Gln Ser Glu Cys Pro Leu Glu Arg Arg Lys Thr Gly Val Phe Thr Val Ala Glu Phe Gly

170 180
Val Gly Leu Arg Thr Lys Val Phe Leu Asp Phe Arg Gln Glu Pro Thr His Glu Cys Asp

190 200
Thr Gly Val Met Gly Ala Ala Val Lys Asn Gly Met Ala Ile His Thr Asp Gln Ser Leu

210 220
Trp Met Arg Ser Met Lys Asn Asp Thr Gly Thr Tyr Ile Val Glu Leu Leu Val Thr Asp

Fig.4(i).

```
                                    230                                 240
Leu Arg Asn Cys Ser Trp Pro Ala Ser His Thr Ile Asp Asn Ala Asp Val Val Asp Ser

                                    250                                 260
Glu Leu Phe Leu Pro Ala Ser Leu Ala Gly Pro Arg Ser Trp Tyr Asn Arg Ile Pro Gly

                                    270                                 280
Tyr Ser Glu Gln Val Lys Gly Pro Trp Lys Tyr Thr Pro Ile Arg Val Ile Arg Glu Glu

                                    290                                 300
Cys Pro Gly Thr Thr Val Thr Ile Asn Ala Lys Cys Asp Lys Arg Gly Ala Ser Val Arg

                                    310                                 320
Ser Thr Thr Glu Ser Gly Lys Val Ile Pro Glu Trp Cys Cys Arg Ala Cys Thr Met Pro

                                    330                                 340
Pro Val Thr Phe Arg Thr Gly Thr Asp Cys Trp Tyr Ala Met Glu Ile Arg Pro Val His
                                COOH-Terminus 1
                                    350                                 360
Asp Gln Gly Gly Leu Val Arg Ser Met Val Val Ala] Asp Asn Gly Glu Leu Leu Ser Glu

                                                                COOH-Terminus 2
                                    370                                 380
Gly Gly Val Pro Gly Ile Val Ala Leu Phe Val Val Leu Glu Tyr Ile Ile Arg Arg Arg]

                                    390                                 400
Pro Ser Thr Gly Thr Thr Val Val Trp Gly Gly Ile Val Val Leu Ala Leu Leu Val Thr
                                    COOH-Terminus 3
                                410
Gly Met Val Arg Ile Glu Ser Leu Val Arg Tyr Val Val Ala]
```

Fig. 4(ii).

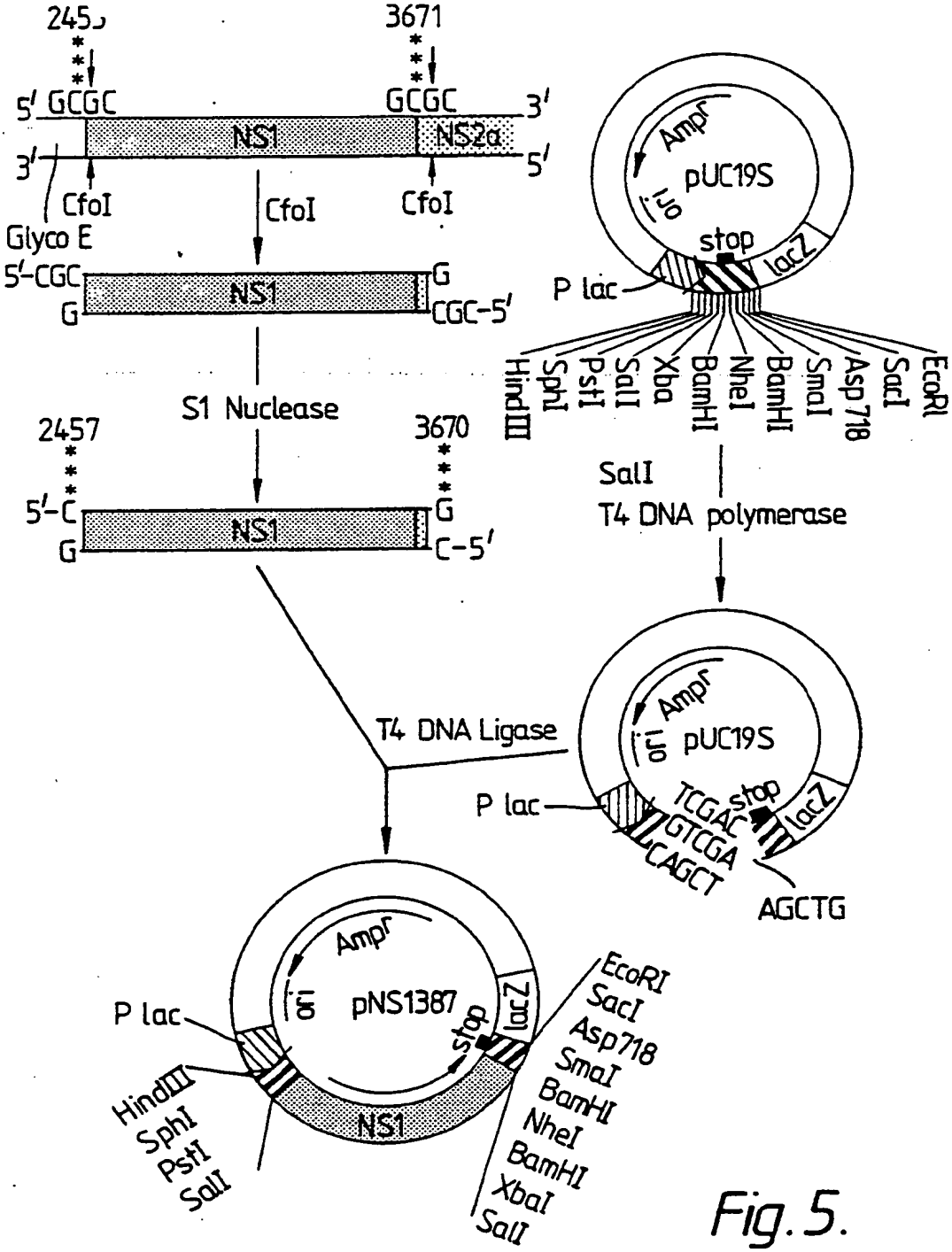


Fig. 5.

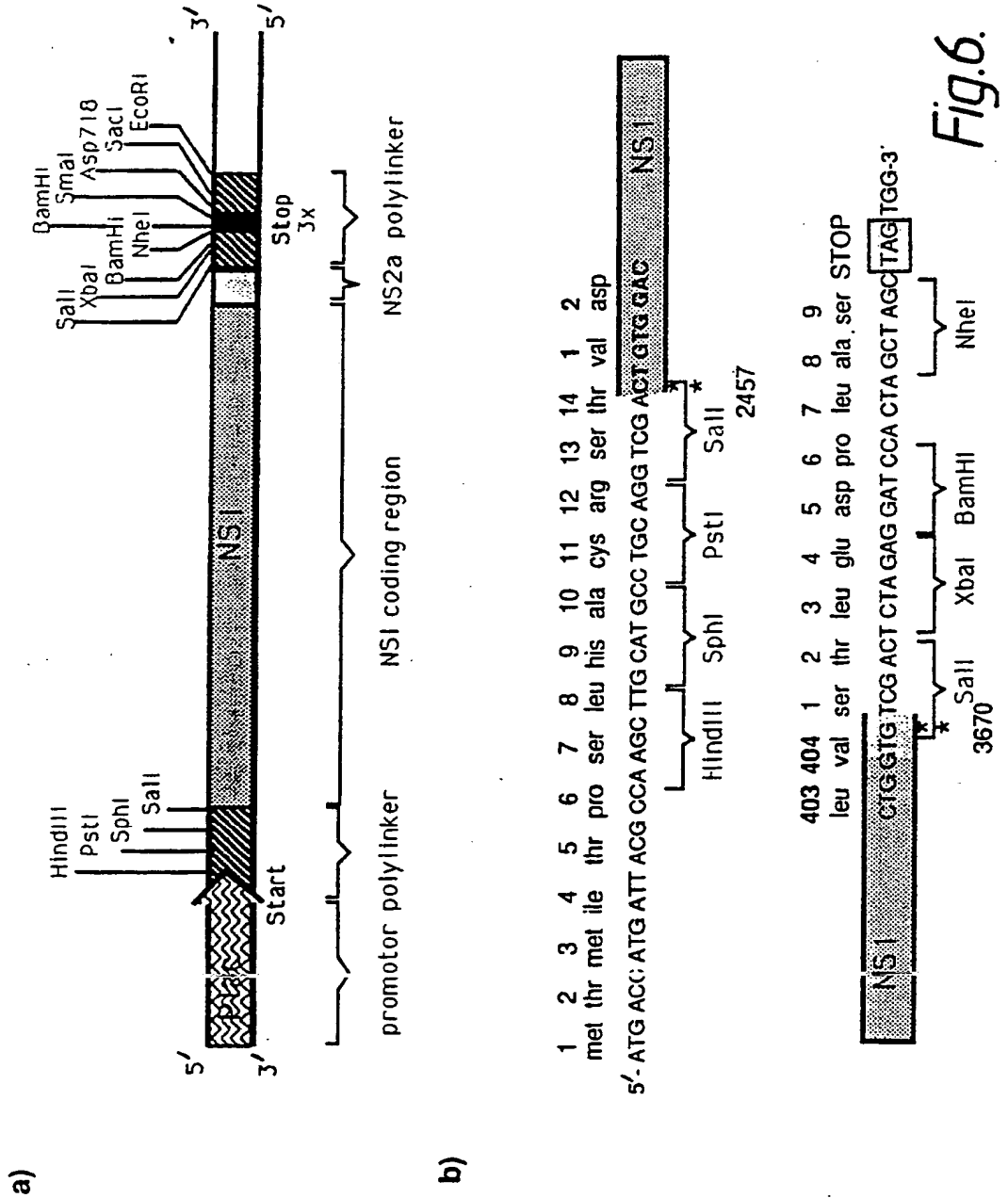


Fig.6.

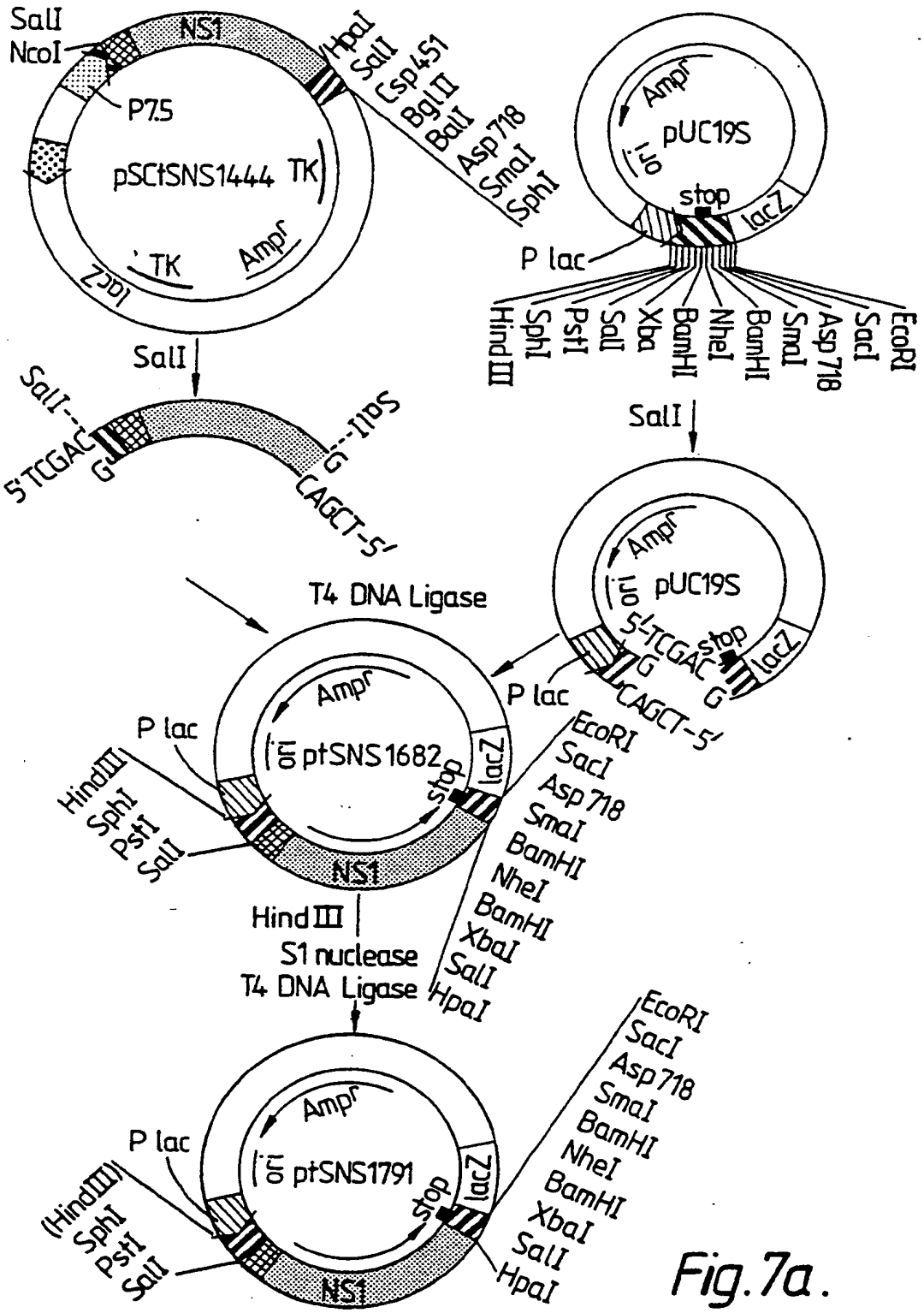


Fig. 7a.

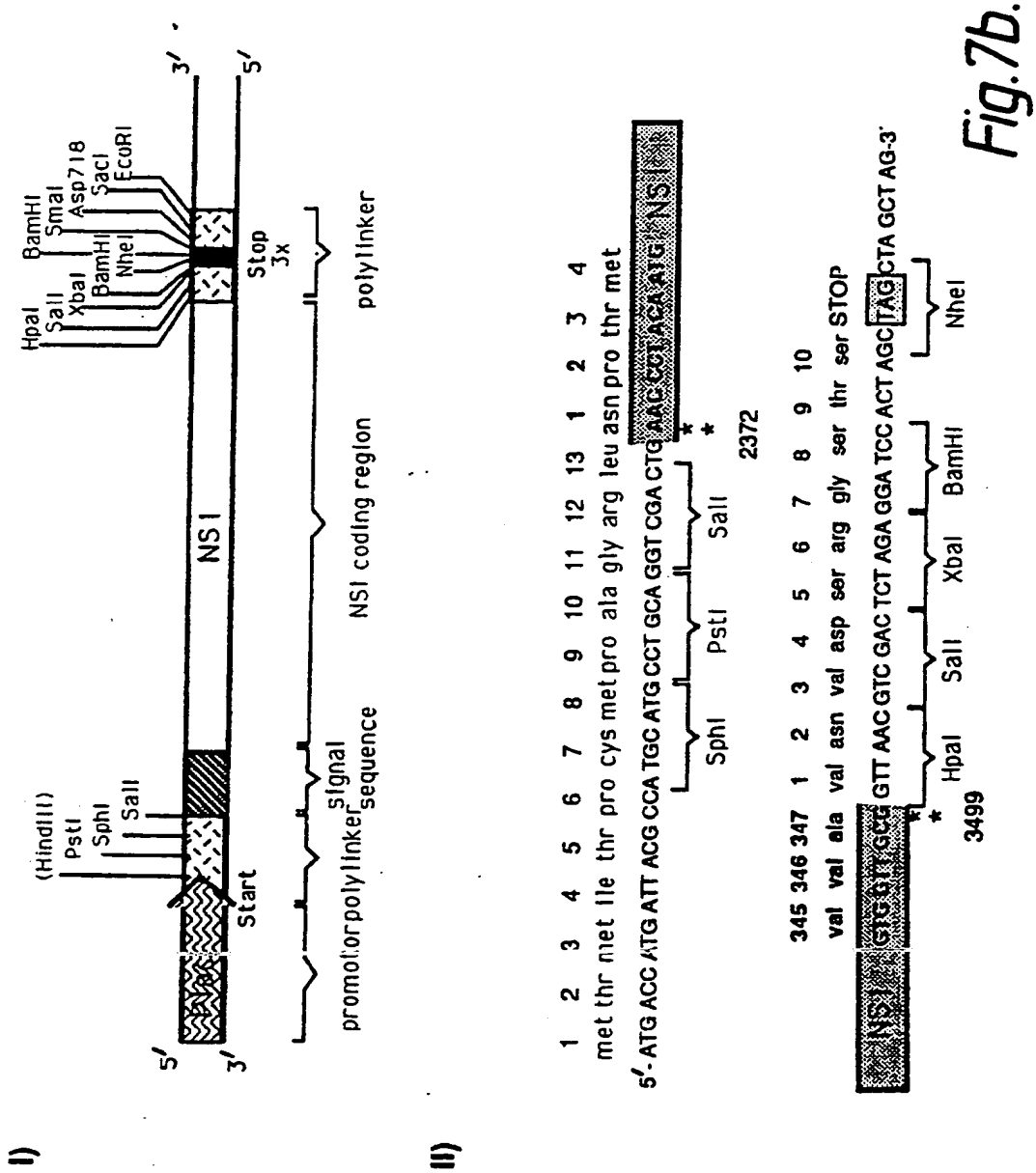


Fig.7b.

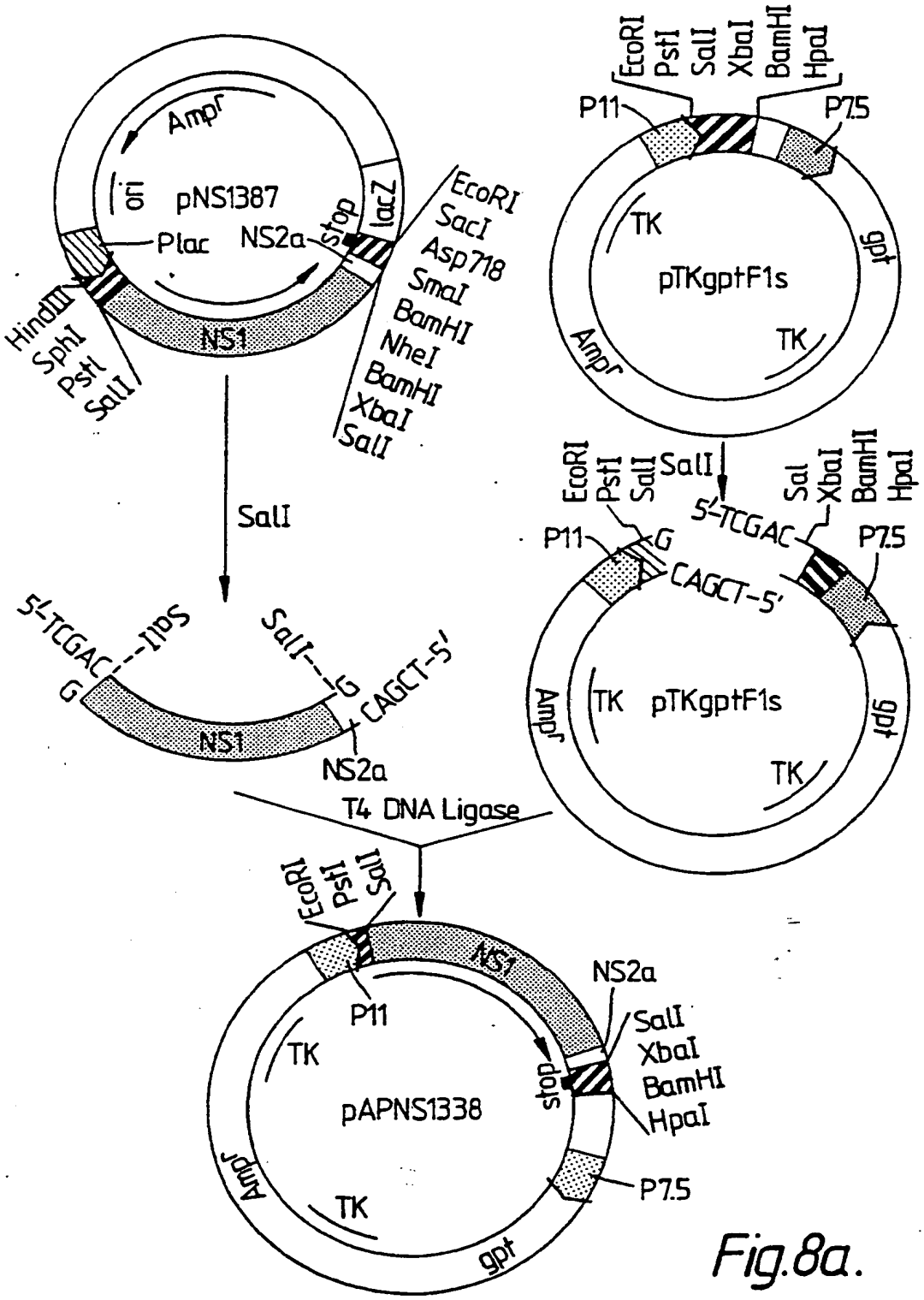


Fig.8a.

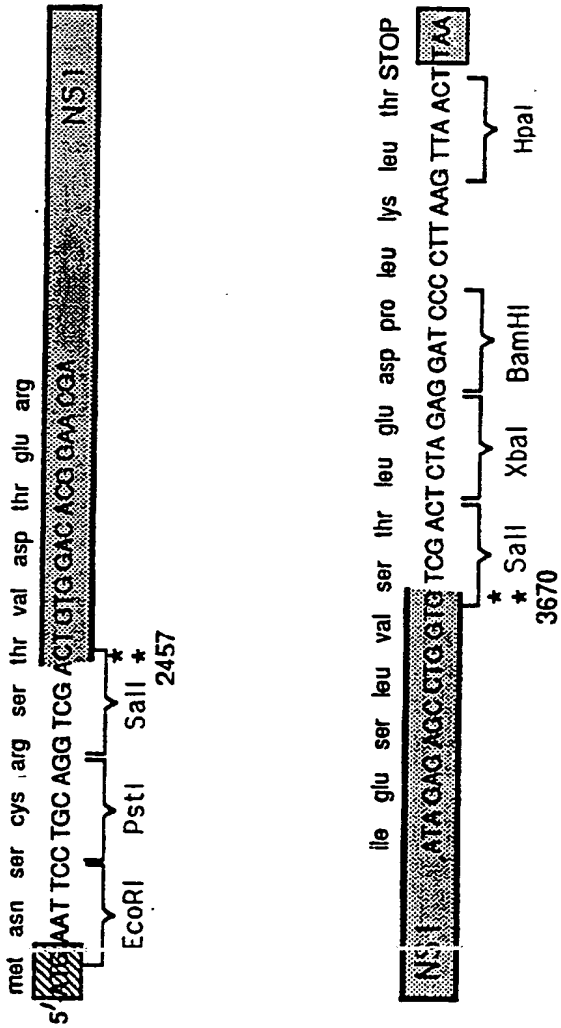


Fig.8b.

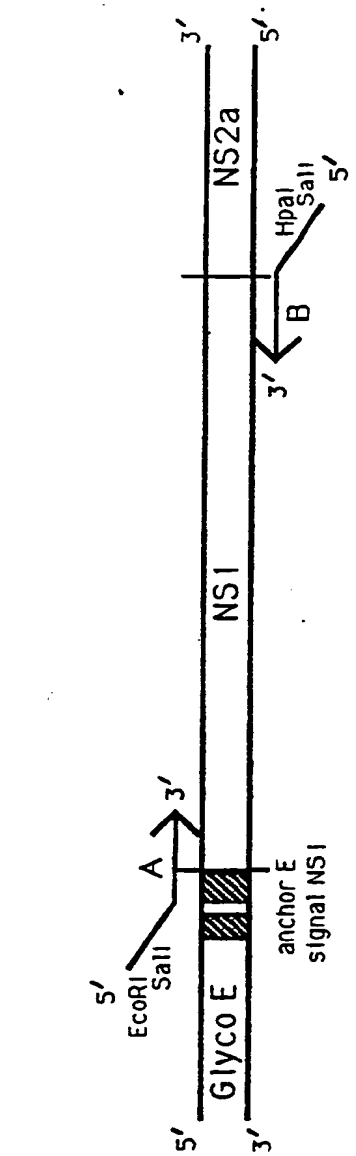


Fig.9.

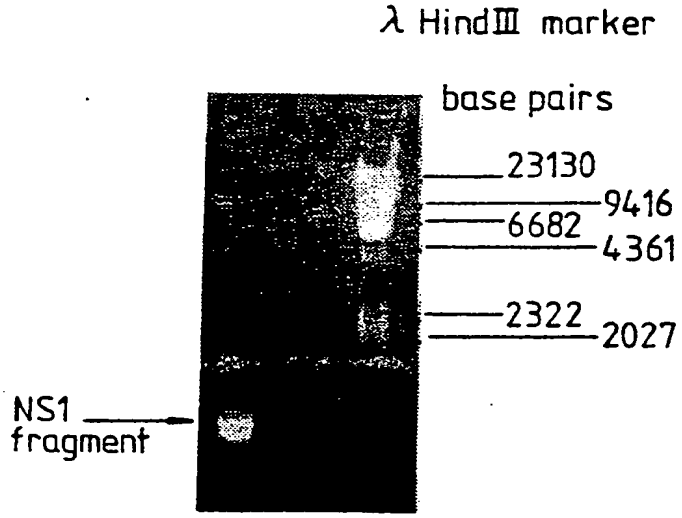

λ HindIII marker
base pairs
23130
9416
6682
4361
2322
2027
NS1
fragment

Fig.10.

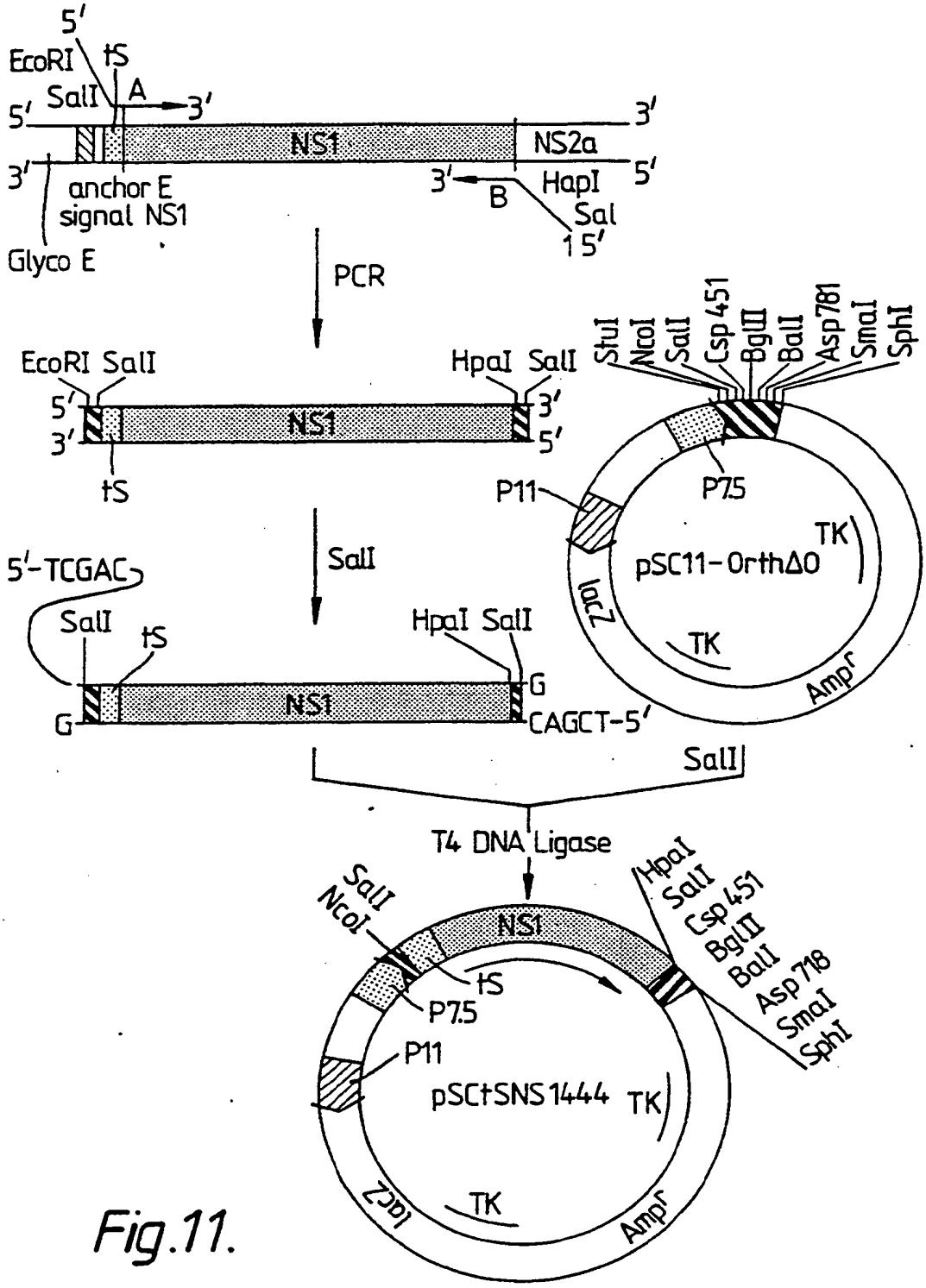


Fig.11.

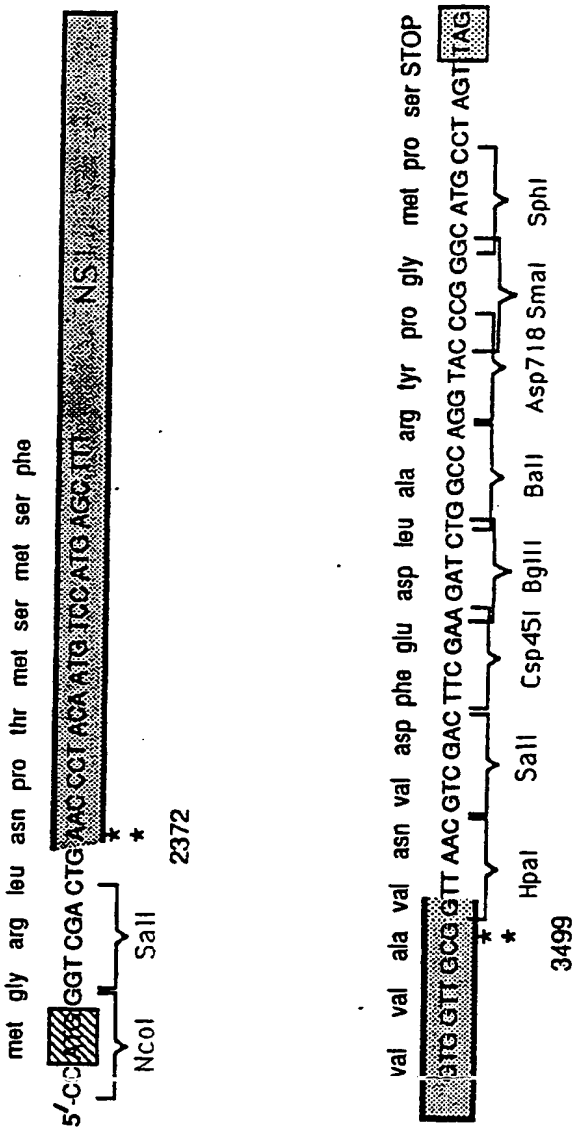


Fig.12.

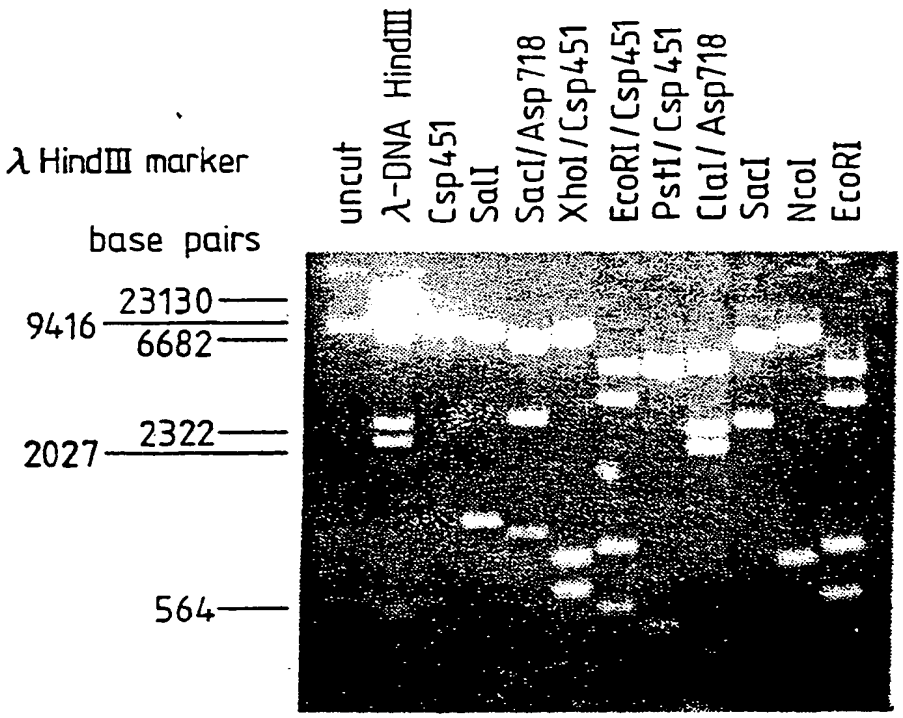


Fig. 13.

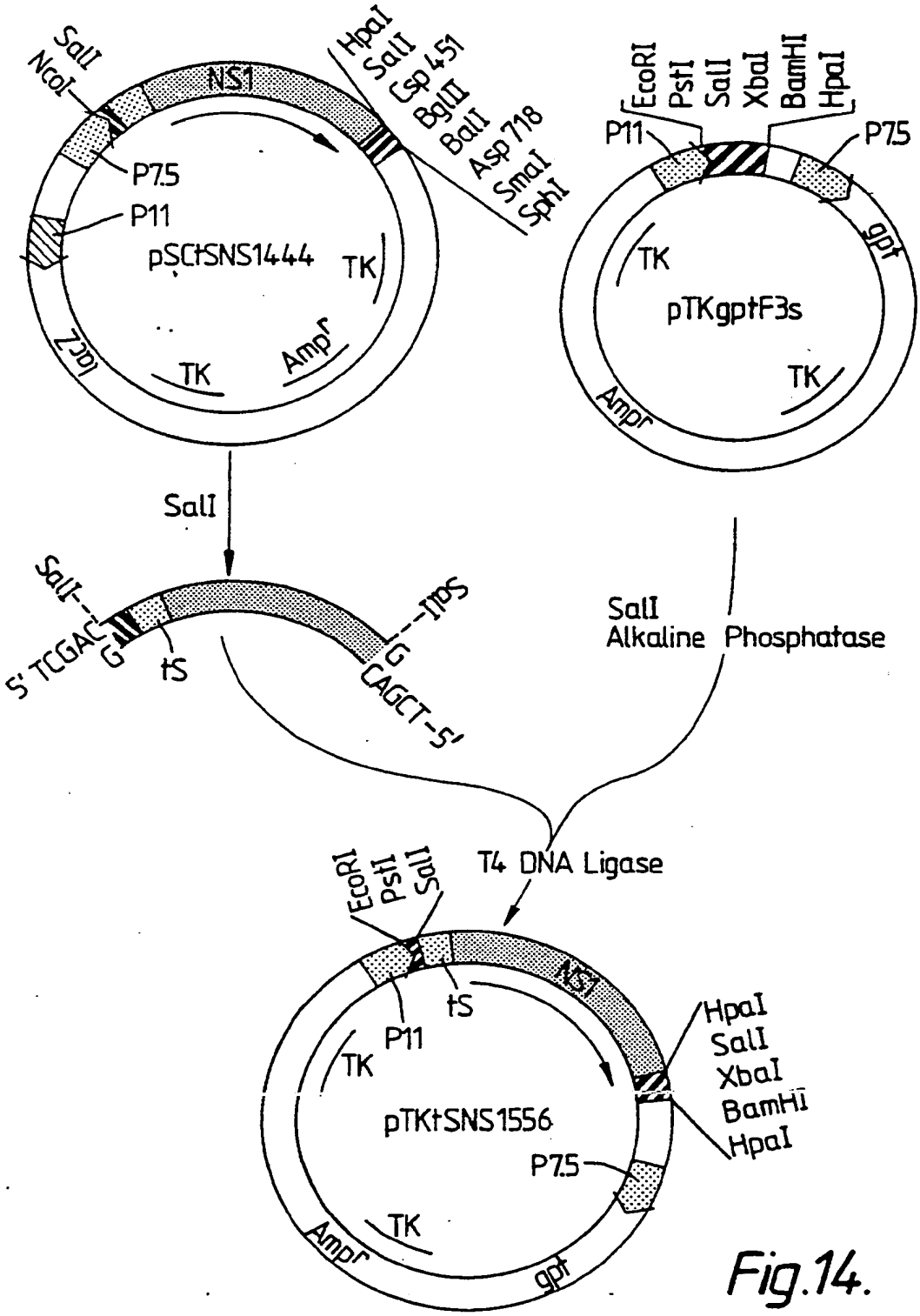


Fig.14.

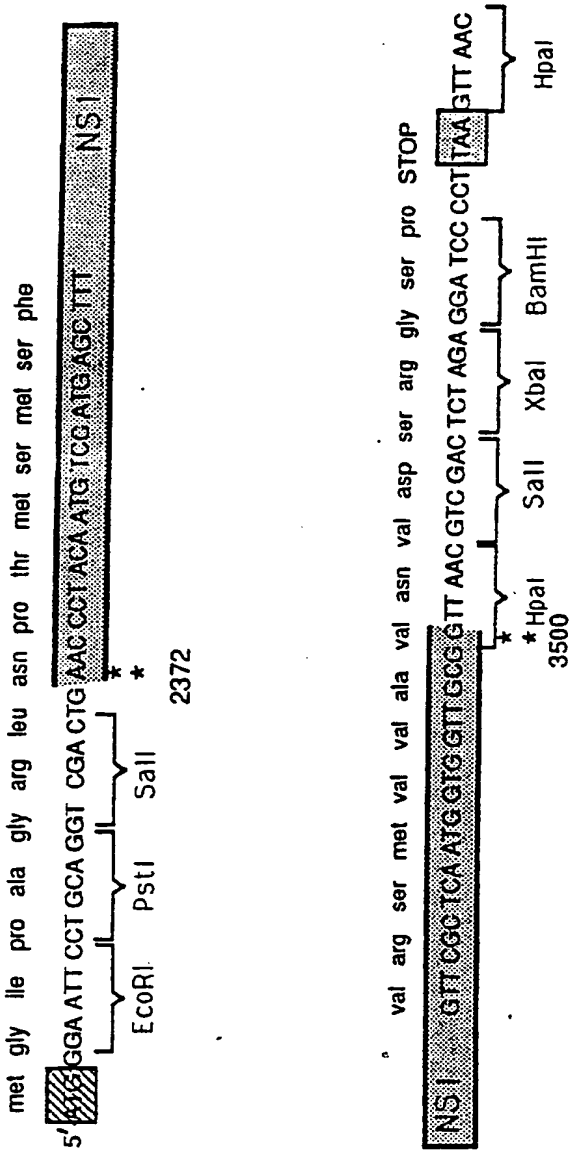


Fig.15.

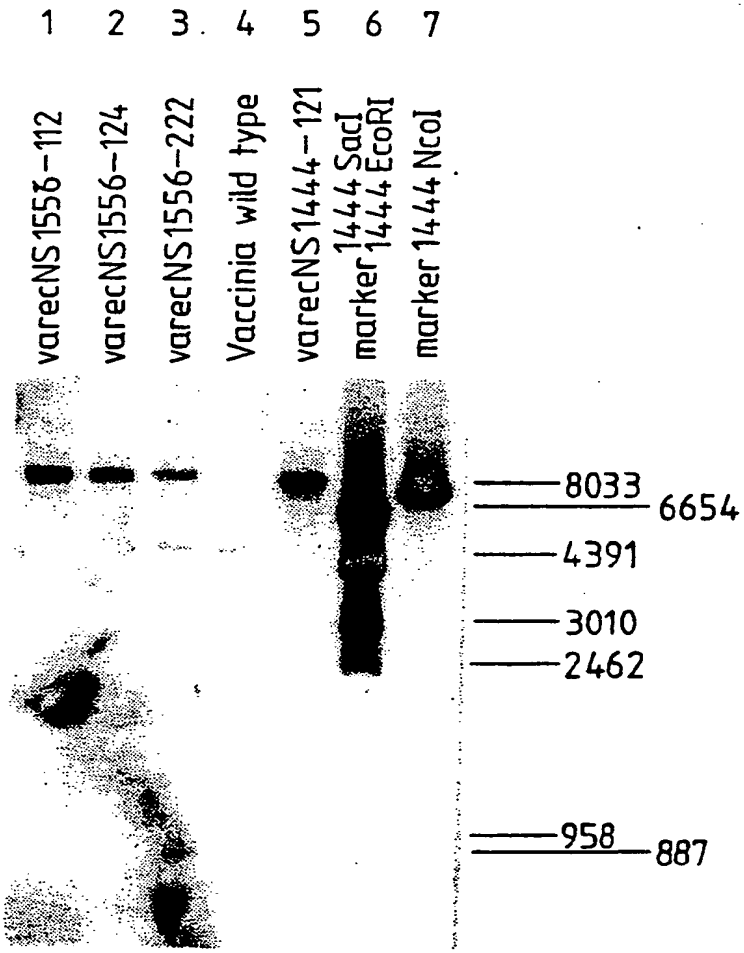


Fig.16.

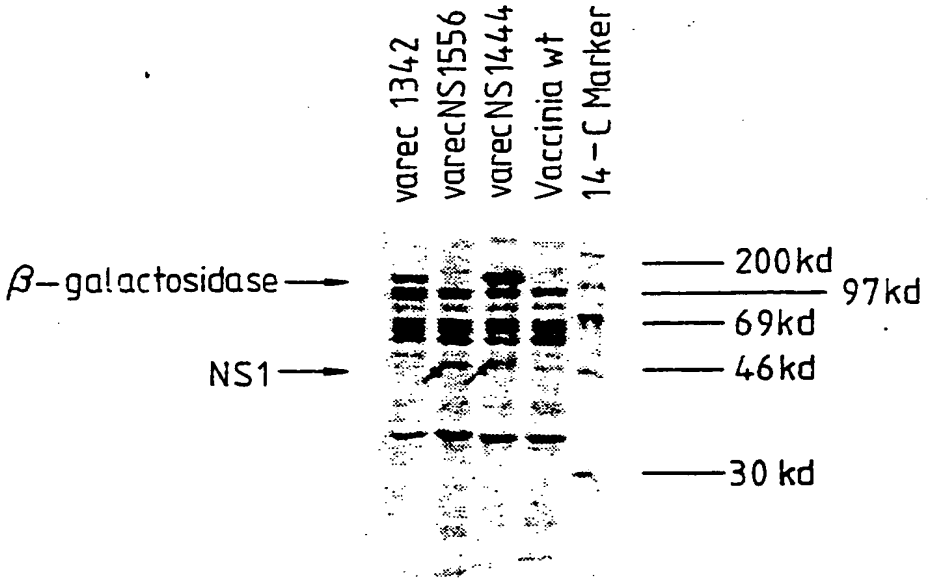


Fig.17.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 30 6154

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,Y	EP-A-0 284 791 (IMMUNO) * Examples 15,16 *	9,17,19,21	
A	VIROLOGY, vol. 168, 1988, pages 197-205, Academic Press Inc.; C.W. MANDL et al.: "Sequence of the structural proteins of tick-borne encephalitis (western subtype) and comparative analysis with other flaviviruses"		
P,X	CHEMICAL ABSTRACTS, vol. 112, no. 21, 21st May 1990, page 203, abstract no. 193124f, Columbus, Ohio, US; A.A. KHROMYKH et al.: "Expression of tick-borne encephalitis virus antigens by various vaccinia virus recombinants", & DOKL. AKAD. NAUK SSSR, 1990, 310(4), 996-9 * Abstract *	16,17,19-21	
P,X	JOURNAL OF CHROMATOGRAPHY, vol. 502, 1990, pages 59-68, Elsevier Science Publishers B.V., Amsterdam, NL; A.J. CROOKS et al.: "Purification and analysis of infectious virions and native non-structural antigens from cells infected with tick-borne encephalitis virus" * The whole document *	1-7	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
E	WO-A-9 001 946 (UNITES STATES) * Claims *	16,17,19-21	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	12-09-1990	SKELLY J.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)